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IMMUNOTOXINS IN HEMATOLOGY

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Ypke Vincentius Johannes Maria van Oosterhout

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Promotor:	**Prof. dr. T.J.M. de Witte**
Co-promotores:	**Dr. F.W.M.B. Preijers**
	Dr. A.V.M.B. Schattenberg
Manuscriptcommissie:	**Prof. dr. P.H.M. de Mulder (voorzitter)**
	Prof. dr. L.A. Monnens
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TABLE OF CONTENTS

SYNOPSIS

Already hundred years ago, Paul Ehrlich envisioned the use of antibodies for the specific targeting of cancer cells without causing harm to healthy tissue. With the invention of the hybridoma technique, Ehrlich's magic bullit concept seemed to come within practical reach. This technique, as described by Kohler and Milstein in 1975, enabled the production of antibodies of defined specificity in sufficient quantities for clinical therapy. As a consequence, a number of clinical trials were initiated, using various monoclonal antibodies (MoAbs) to target diffuse and solid tumors and varying immune diseases.

OKT3 was the first therapeutic MoAb to be approved for marketing by the US Food and Drug Administration (FDA) in 1986. This murine MoAb demonstrated activity in reversing acute organ transplant rejection. MoAb 17-1A, registered in 1994 in Germany, was the first MoAb for the treatment of cancer to win approval in any country. This murine MoAb significantly reduced post-operative mortality in colon cancer patients when applied as adjuvant therapy. Unfortunately, these two cases appeared to be exceptions rather than the forebode of a new class of broadly effective therapeutics. In general, responses to MoAb therapy were poor, if any, and their administration resulted frequently in allergic type reactions. Excuses were found in the immunogenicity of the xenogenic MoAbs, the heterogenic expression or modulation of antigen, insufficient accessibility of target cells, the presence of MoAb-capturing circulating free antigen, and limited interaction of the (mostly murine) MoAbs with the host immune effector mechanisms.

Though far from satisfying, these initial experiences formed the base for the development of improved antibody strategies. These include the (partial) substitution of the constant regions of the murine MoAbs with their human counterparts to render them less immunogenic and to improve their interaction with the host immune system. More recently, different techniques have become available which enable the production of fully human MoAbs or fragments thereof. Another strategy forms the generation of bispecific antibodies with affinity for both the target cell and an immune effector cell. By bridging these cells, bispecific antibodies aim at the maximal exploitation of the patient's immune system. As an alternative, MoAbs have been conjugated to effector molecules, like radioisotopes or plant/bacterial derived toxins, in an attempt to improve on their intrinsic, host independent, cytotoxicity.

The implication of these technological and conceptual advances strongly boosted the scientific as well as pharmaceutical interest. In line with this MoAb-revival, the FDA approved its first therapeutic antibody for treatment of cancer in 1997. This humanized CD20 MoAb (Rituximab) demonstrated efficacy in the treatment of certain forms of B-

cell Non-Hodgkin's lymphoma. Since then, an additional five (partly) humanized MoAbs have been approved by the FDA for the treatment of certain forms of cancer and immune disorders. Moreover, with about 60 MoAb-based agents currently being tested in the clinic, MoAb represent the second largest category of biotechnology products in clinical trials.[1]

The present thesis focuses on the approach of antibody-toxin conjugates, the so called immunotoxins (IT). More specifically, the potential pitfalls, as well as the drawbacks and therapeutic advantages of these agents when applied in the field of hematology will be highlighted.

REFERENCE

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CHAPTER 1

IMMUNOTOXINS, GENERAL INTRODUCTION

CONTENTS

1.1 GENERAL PRINCIPLE

Conventional ITs consist of MoAbs conjugated to a potent toxin, mostly of bacterial or plant origin. Their mechanism of action is depicted in Figure 1. The MoAb-moiety first binds specifically to its antigen expressed on target cells, following which the entire antigen-IT complex is internalized. Once inside the cell, the bond between the MoAb and toxin is broken, thereby releasing free toxin into the cytoplasm. The toxin then irreversibly inhibits protein synthesis by means of a catalytic reaction culminating in cell death. Because the toxin-moiety is unable to enter the cell autonomously, and is inactive outside the cell, ITs are only hazardous to cells capable of binding and internalizing the MoAb.

Instead of MoAbs, or fragments thereof, cells can also be targeted by cytokines which receptor is (over)expressed on the target cell. As an alternative to chemical linkage, MoAb-fragments or cytokines can also be genetically spliced to a toxin, generating a recombinant IT.

Figure 1 *Mechanism of action*

1.2 MONOCLONAL ANTIBODY PART

Antibody structure

Antibodies can be considered as bifunctional molecules, compromising a highly variable antigen-binding domain (V domain) on one site and a constant effector domain (C domain) on the other site (Figure 2). Based on their physical properties, antibodies can be divided in IgM, IgG, IgA, IgE, an IgD classes, each having its own function in the host defense system. IgG is the antibody class most widely exploited for clinical use. The IgG molecule compromises two heavy chains of the structure VH-CH1-CH2-CH3 and two light chains of the structure VL-CL. The heavy and light chain are linked to each other by a disulphide bond between CH1 and CL. Additional disulphide bonds formed in the so called hinge region of the two heavy chains result in the formation of homodimers, containing two identical antigen binding-sites. The actual antigen-recognition site is provided by the complementarity-determining regions (CDR), formed by three hypervariable loops of each the VH and VL domain (CDRH1-3 and CDRL1-3, respectively). This antigen-combining region is immunologically defined as the antibody's idiotype. The IgG effector functions are located in the Fc-part in the form of an C1q complement-binding site and Fc-receptor binding site.

When considering MoAbs solely as a toxin transporter, it makes sense to use only those fragments containing the binding function of the CDR's, such as the 110 kDa bivalent $F(ab')_2$ fragment, or the monovalent Fab' or Fab fragments (Figure 2). The even smaller recombinant single-chain Fv fragments (sFv) contain only the V_L and V_H domain connected to each other through a short flexible peptide spacer. In disulfide stabilized Fv fragments (dsFv), the peptide linker is replaced by a more stable disulfide bond introduced by genetically engineering cysteine residues in V_L and V_H at positions allowing post-translational disulfide bond formation. The Fv-fragments are only 26 kDa in size, less than 20% of the 155 kDa IgG.

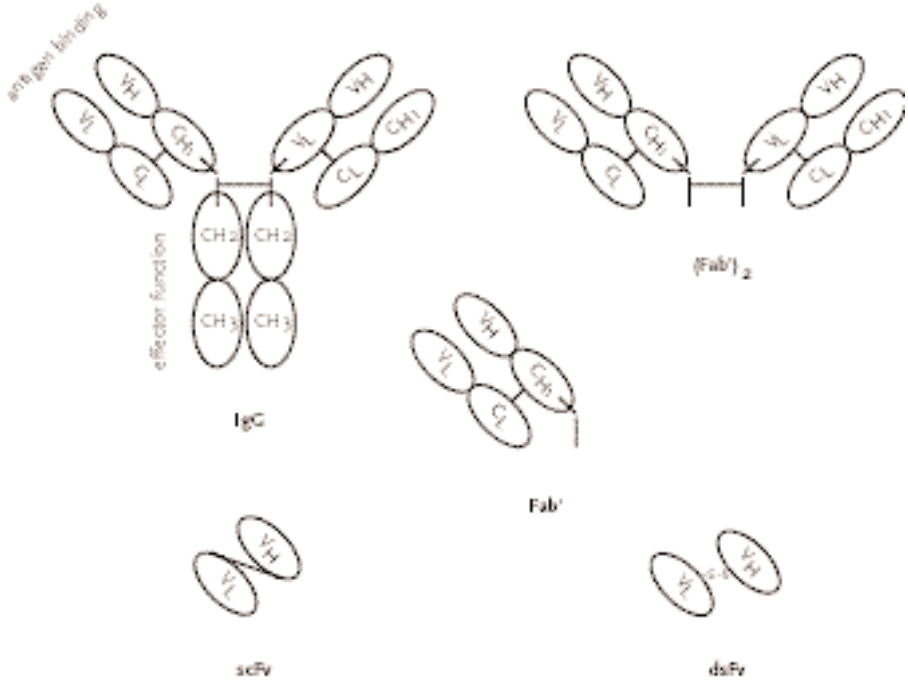

Figure 2 *Whole IgG and IgG-derived fragments used in Immunotoxins*

Removing the Fc portion of a 155 kDa IgG molecule results in F(ab')$_2$ fragments of 110 kDa. These can be converted to Fab' fragments by reduction, proteolytic treatment or by recombinant means. Single-chain Fv (scFv) fragments consists of the variable domain of a MoAb joined by a linker(26 kDa). In a dsFV the linker is replaced by a more stable disulfide bond.

Of note, in particular cases, the MoAb Fc-part can be used as the targeting moiety. An example forms the treatment of allergic disorders by the elimination of high-affinity Fc-epsilon-receptor I (FcγRI) expressing mast cells and basophils by toxin-conjugated IgE Fc-fragments.[1-3]

Specificity and distibution of antigen

The MoAb's binding specificity is the major property determining its suitability as a toxin transporter. Ideally, the MoAb binds an antigen that is expressed solely on those cells that are to be eliminated. The Ig-idiotype expressed by malignant B cells and myeloma cells, for instance, represents a tumor-clone specific marker.[4-9] A more general tumor specific marker is formed by the mutant epidermal growth factor receptor (EGFRvIII), expressed in glioblastoma, and carcinomas of breast and lung. EGFRvIII contains an in-frame deletion resulting in the surface expression of a tumor-specific extracellular sequence. ITs directed against this tumor-specific target demonstrated

clear cytotoxicity to EGFRvIII transfected cells, while showing little or no efficay towards cells expressing high levels of normal EGFR.[10,11]

In practice, however, tumor restricted antigens are rare and differences in expression relative to non-target tissue are generally quantitative rather than qualitative (*e.g.* tissue-associated rather than tumor specific). About 25-30% of patients with breast and ovarian cancer shows a 10 to 100-fold overexpression of the human epidermal growth factor receptor 2 (HER-2, ebrB2). This phenotype, caused by a mutation in the HER-2/neu gene, is associated with a rapid disease progression and a poor prognosis.[12] Treatment of this particular patient group with anti-HER-2 MoAb resulted in objective responses.[13] Several groups are now working on anti-HER-2 ITs in an attempt to further improve on the clinical efficacy.[14-20] Other tumor-associated markers which have been clinically exploited as target for ITs include lewis^y (Le^y) expressed on a variety of solid tumors[21], high-molecular-weight melanoma associated antigen (HMW-MAA) expressed on melanoma[22-26], CD56 (neural cell adhesion molecule, NCAM) associated with small cell lung cancer[27-29], and carcinoembryonic antigen (CEA) overexpressed on adenocarcinomas[30]. The clinical use of ITs directed against Le^y clearly illustrated the potential limitations of targeting tumor-associated antigens.[21] The Le^y-expression on normal vascular endothelial cells resulted in serious vascular leak syndrome (VLS) preventing the administration of sufficient IT to achieve therapeutic blood levels.[31] Similarly, the HER-2 expression on normal liver cells caused hepatotoxicity in all six patients treated with an anti-HER-2 IT.[20] Generally, though, it has been demonstrated that ITs can be delivered safely to tumor-associated antigens without severe irreversible toxicities occurring. Moreover, target-antigen expression on healthy bystander tissue is less critical in case of strongly regenerative tissue. An illustrative example form the hematopoietic lineage markers.[32] Though an effective therapy is expected to affect healthy lineage cells as well, early progenitor cells will rapidly restore the hematopoietic compartment. At current, hematological disorders represent the group most effectively targeted with immuntoxins (see also Chapter 2).

The opposite concern of cross-reactivity is the occurrence of antigen negative (or non-overexpressing) target cells. This can be at the population level, *e.g.* less than 30% of patients with breast/ovarian cancer over-expresses HER-2, but may also be at the patient level, due to heterogeneous antigen expression within the tumor.[33-35] In practice, an arbitrary threshold is often set defining the minimal percentage of antigen-positive cells required for therapy. In certain cases, co-administration of cytokines may increase the expression of tumor-associated antigens. An example forms the IFN-γ induced upregulation of the tumor-associated antigen 72 (TAG-72) and CEA on colon carcinoma.[36-39] Moreover, the use of an appropriate combination of immunotoxins may circumvent the heterogeneity observed in the expression of individual antigens.[40-44] Additional obstacles preventing an optimal targeting are a concentration of natural ligand,

competing with the MoAb for binding, or a high amount of circulating free antigen or antigen expressing normal cells, which may both act as an antigen sink.[45,46]

As an attractive alternative to direct tumor-targeting, Burrow *et al.* introduced the concept of targeting the tumor-nursing vascular bed.[47-49] This approach affords several important advantages: (1) the pivotal problem of poor tumor penetration is circumvented, (2) even incomplete destruction of the vascular bed results in massive tumor destruction, and (3) a single immunotoxin can be used for the treatment of a variety of tumors of different origin. The proof of principle was generated in nude mice grafted with IFN-γ transfected tumor cells. These cells induced an artificial expression of major histocompatibility complex (MHC) Class II antigens on the surrounding tumor vascular endothelium. The subsequent administration of anti-Class II directed IT caused extensive thrombosis of the tumor vasculature, inducing permanent complete remissions in over half of the animals. In later trials, comparable results could be obtained using ITs reactive with endoglin, a natural proliferation-associated antigen of endothelial cells.[50-52]. More recently, 'clotting initiating human tissue factor' proved in this setting to be a useful alternative for the conventional plant or bacterial derived (immunogenic) toxin-moiety.[53-55]

Internalization of MoAb-antigen complex

Following antigen-binding, the toxin-conjugated MoAbs needs to deliver the toxin to the cytoplasm. Preijers *et al.* demonstrated for several anti-T-cell ITs that the absolute amount of internalized molecules rather than the antigen surface expression determines the efficacy.[56] The amount and kinetics of internalization itself are determined by the combination of antigen, MoAb and target cell.[56-67]

Surface antigens differ widely regarding their intrinsic internalization capacity. Some appear to be good internalizers, like T-cell antigens CD3 and CD7 and B-cell antigen CD22, while others hardly internalize, like T-cell antigen CD8 or B-cell antigen CD20.[56,68-71] The use of MoAb-fragments instead of whole IgG generally reduces the efficacy of the respective IT, either due to a decrease in antigen-binding or in the subsequent internalization.[66,72-75] Anti-CD5 ITs form an exception and are more effective with $F(ab')_2$ or Fab'-fragments than with whole IgG.[65,67,76] For CD19-ITs it has been demonstrated that the MoAb isotype may affect the internalization and, thereby, the efficacy of the IT. This phenomenon could be attributed to an interaction of the Fc-part of murine IgG1 MoAb (and not IgG2a MoAb) to the Fc-gamma-receptor IIa (FcγRIIa). Analogous to the mechanism as described by Kurlander, this facilitated the internalization of antigen-bound IT.[77]

Once internalized, the intracellular routing determines the ultimate efficacy of the immunotoxin.[59,69,78,79] The IT needs to be transported to an appropriate subcellular compartment to facilitate the translocation of the toxin-moiety to the cytoplasm. As a result, ITs with comparable internalization rates may differ greatly in efficacy. Anti-CD19

ITs, for example, are generally less effective than anti-CD22 ITs, despite similar or higher internalization rates.[80-82] For T-cell antigen CD2, and the B-cell antigen surface-IgD, it has been demonstrated that MoAb binding epitopes closer to the cell surface make superior ITs. This was also attributed to a favorable intracellular handling.[59,62]

The above picture becomes even more complex considering that plant and bacterial toxins prefer different routes of internalization for optimal efficacy (see Paragraph 1.3).[58,83] Therefore, an indirect sandwich cytotoxicity assay has been developed which facilitates the *in vitro* screening of particular MoAb-toxin conjugates.[84] In this assay, dilutions of MoAbs are incubated with the target cells, followed by the binding of a second (isotype-specific) antibody-fragment conjugated to a toxin of choice. This technique allows the quick screening of a panel of MoAbs in combination with different toxins.[84-87] The only concern is that the indirect approach might influence the internalization and/or intracellular handling of the particular toxin. Using this assay, we dramatically underscored the potential of a CD7 MoAb that proved highly effective when conjugated directly to the toxin applied (WT1-dgA, unpublished observations).

1.3 TOXINS

The toxins most commonly applied for the construction of ITs are derived from either bacteria or plants and have in common that they catalytically and irreversibly disrupt protein synthesis, typically at picomolar concentration (Figure 3). The bacterial toxins most extensively studied so far are *Pseudomonas* exotoxin (PE) and diphtheria toxin

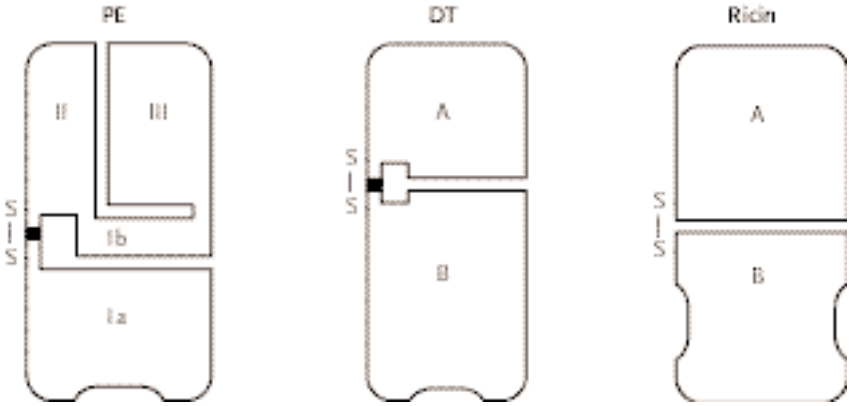

Figure 3 *Schematic representation of toxin molecular structure.*

PE, Pseudomonas exotoxin: Ia,Ib - binding domain, II - translocation domain, III - catalytic domain. DT, diptheria toxin: A, catalytic fragment, B, binding fragment. The shaded segments mark the position of the protease-sensitive loop region. Ricin: A, active chain, B, binding chain.
Figure adapted from E.J.Wawrzynczak.[277]

(DT). The plant toxins, also referred to as ribosome inhibitory proteins (RIP), can be divided into holotoxins (type II RIP), including abrin, mistletoe lectin, modeccin, and ricin, and hemitoxins (type I RIP), such as gelonin, pokeweed antiviral protein (PAP), and saporin.

Pseudomonas exotoxin

PE contains three functional domains, responsible for binding (domain Ia), translocation (domain II), and protein synthesis inhibition (domain III). Domain Ib, of yet unknown function, joins domain II and the C-terminal domain III.[88-90] Native PE is excreted by the bacterium as a 66 kDa single-chain protein. The N-terminal domain Ia binds the α2-macroglobulin receptor present on the surface of all eukaryotic cell types. Following endocytosis, domain II is proteolytically cleaved at a low pH by cellular protease furine, resulting in two disulfide-bonded fragments of 28 and 37 kDa.[91,92] The 37 kDa fragment contains part of the translocation domain II and the active enzyme domain III. The latter contains a REDLK sequence which facilitates the transport of the 37 kDa fragment from the transreticular Golgi apparatus to the endoplasmatic reticulum (ER).[93] There, the remaining domain II fragment mediates the translocation of domain III to the cytoplasm. In the cytoplasm, domain III inactivates elongation factor 2 (EF-2) by its ADP ribosylating activity resulting in the cell's death. Current PE-based IT use truncated forms of PE, in which the entire Ia domain (PE40)[94-97] and frequently also part of the Ib domain (PE38, PE35)[98-105] have been deleted. Cell targeting ligands are chemically or genetically linked to the N-terminus. In some cases, the C-terminal REDLK sequence is altered to a KDEL sequence.[106-109] This sequence has a higher affinity for the ER KDEL receptor and, therefore, results in an increased efficacy of the respective IT.

Diptheria toxin

DT compromises an A and B-domain, which are responsible for cell binding and inhibition of proteins synthesis, respectively.[88] The crystal structure reveals a third domain, the translocation domain (T), located within the B-domain in the center of the molecule.[110] Native DT is released in the bacterial medium as a single polypeptide chain of 535 amino acids of approximately 62 kDa. Outside the cell, DT is proteolytically cleaved between the C-terminal A (24 kDa) and N-terminal B-domain (38 kDa), resulting in a disulfide bonded two-chain form. The C-terminal B-domain binds to a complex of heparin-binding EGF-like growth factor precursor and CD9. After endocytosis, the disulfide bond is broken and the low endosomal pH promotes a conformational change of the T domain resulting in the formation of a transmembrane channel through which the enzymatic A domain can enter the cytoplasm.[111,112] In the cytosol, the A domain disrupts cellular protein synthesis by ADP ribosylation of EF-2. For the construction of IT, the C-terminus of either a binding site mutant (a.o. CRM107, CRM9)[113,114] or C-terminal truncation mutant (DT388, DT390, DAB486, DAB389)[115-118] is linked to a cell binding ligand. Because most people are vaccinated against DT, C-terminal truncation

mutants are to be preferred as pre-existing anti-DT antibodies may primarily be directed against the cell binding B-domain.[118]

Plant holotoxins toxins

Plant holotoxins compromise two disulfide-bonded chains: a protein synthesis inhibiting A-chain, and a cell binding and translocation facilitating B-chain.[119] Ricin, a natural product derived from the castor bean *(Ricinus Communis)*, is the plant holotoxin most extensively tested *in vivo*. Native ricin is synthesized as a 64 kDa single chain proenzyme containing a 12 residue linking peptide joining the 32 kDa A- and B-chain. Following post-translational proteolytic cleavage, an interchain disulfide bond links the two chains. Cellular intoxication starts by binding of cell surface galactose residues by the three lectin domains within the B-chain.[120] After endocytosis, a considerable portion of the internalized ricin is transported and degraded in the lysosomes.[121] A fraction of the internalized toxin is delivered to the trans-Golgi network. The further routing appears to be dependent on receptors recycling between the terminal compartments of the Golgi stack and the ER.[122-124] The B chain dissociates from the A chain somewhere during the transport to the ER. In the ER, the A chain is translocated to the cytoplasm and inhibits protein synthesis through N-glycosidase activity removing the base of A^{4324} in 28s rRNA, thus preventing the association of EF-1 and EF-2.[125]

For clinical use, the binding capacity of the B-chain has to be eliminated either by addition of excess galactose or lactose[126] (only for *ex vivo* applications), chemical blocking of the lectin-sites *e.g.* with galactose-based affinity labels (blocked ricin)[127-131], or omitting the entire B-chain[132]. An additional concern forms the high mannose and fucose carbohydrate groups present on A and B-chain, which are bound by receptors on liver Kupfer cells leading to rapid clearance and hepatoxicity.[133] Using either chemically deglycosylated ricin A (dgA) or non-glycosylated recombinant ricin A (rA) has successfully circumvented this problem.[134-137] Notably, deglycosylation is not an option for blocked ricin due to the galactose-based blocking of lectin activity.

The cytotoxicity of ricin A-based ITs is substantially lower than that of blocked ricin-based ITs.[128,138] This can largely be ascribed to the membrane translocation enhancing capability of the B-chain.[129,131,139] Moreover, the B-chain seems to protect the A-chain from intracellular degradation.[140] Whereas PE and DT are dependent on proteolytic cleavage for enzymatic activity, the ricin A chain is largely inactivated by endosomal or lysosomal proteases. Compounds that neutralize the low pH of intracellular compartments, or interfere with the routing to the lysosomes, may strongly enhance the cytotoxicity of ricin A-based IT.[141-143] Most commonly used are lysosomotropic agents, like NH_4Cl and chloroquine, and carboxylic ionophores, like monensin and nigericin.[68,144-150] Though biologically active concentrations are usually not achievable *in vivo*, these agents have proven to be useful for *ex vivo* applications like the purging of hematopoietic stem cell grafts.[151-153]

Plant Hemitoxins

Plant RIP I Hemitoxins, like saporin, gelonin, and pokeweek antiviral protein (PAP), contain no cell binding domain and resemble the A-chain of RIP II in size and in function.[119] For the construction of ITs, RIP I are typically conjugated directly to a cell targeting ligand without further modifications.[154-158]

Alternative toxins

Li and Ramakrishnan constructed a recombinant hybrid plant-bacterial toxin by fusing the coding region of ricin A-chain to the A-domain of DT. The rationale is that since bacterial and plant toxin act at different steps in protein translocation, a combination of their activities could be more effective. In fact, ITs containing these chimeric toxins were *in vitro* 100-1000 fold more effective than ITs prepared with either toxin alone.[159]

Though extremely potent, the major drawback of the bacterial and plant derived toxins is their proven immunogenicity. Especially now human targeting ligands (antibodies and cytokines) are readily available, it makes sense to search for human toxin equivalents as well. So far, several human Rnases, Dnases, and human TNFα have been used to construct fully human ITs.[160-172] Though not as toxic as their plant/bacterial counterparts, the initial *in vitro* results appear to be encouraging. The major benefit of human ITs is to be expected in the treatment of diseases which ask for successive administrations (*e.g.* autoimmune disorders). Since they have not been clinically tested yet, it remains an open question whether the junction of human toxin and cell targeting ligand does not form a new antigenic determinant.

As an alternative to the large catalytic toxins (typically ≥30 kDa), several synthetic chemotherapeutic molecules have also been coupled to MoAbs. These include vinblastine, doxorubicin, daunomycin, methotrexate, adriamycin, maytansinoids and calicheamicin.[173-182] Due to their smaller size (<1 kDa), these agents may improve tumor tissue distribution and will probably be less immunogenic. As compared to their unconjugated forms, these agents proved more effective, and cause less side effects, following conjugation to the MoAb. Their intracellular mechanism of action, however, is identical. As such, they lack the advantage of plant or bacterial derived toxins which, by there alternative mode of cytoxicity, may overcome acquired drug resistance to conventional therapy.[183-189]

1.4 LINKING MONOCLONAL ANTIBODY AND TOXIN

Chemical conjugates

Conventional ITs are prepared by linking a toxin fragment and cell binding ligand using a chemical crosslinker. Early studies demonstrated that plant toxin-based ITs should be conjugated by a disulfide-bond containing linker to enable the essential intracellular dissociation of ligand and toxin.[126,190-192] For maximal stability outside the cell, crosslink-

ers have been developed that contain a benzene ring and methyl group around the disulfide bond to protect it from reduction by thiols in the blood and tissues.[193,194] When using blocked ricin, a nonreducible thioether bond is prefered as the natural occurring interchain disulfide bond is already present. The same holds true for truncated bacterial toxins, still containing their native protease-sensitive sequence and interchain disulfide bond.

The chemical conjugation procedure has some potential disadvantages. The ligand conjugation is based on the derivatization of lysine residues, some of which may be located within the antigen binding domain. The same holds true for derivatization of the toxin, which may interfere with its catalytic activity.[195] In general, chemically constructed ITs form a heterogeneous mixture with respect to derivatization sites and the number of toxins per IT molecule. Recently, production methods have been developed resulting in an increased product uniformity.[196] There is no clear consensus, though, regarding the optimal number of conjugated toxins per ligand.[81,197-200] ITs should be individually tested to determine whether the addition of two versus one toxin improves the *in vivo* cytotoxicity. If a ligand-toxin combination forms an effective conjugate, the conventional chemical production method has proven to be straightforward with good production yields, especially for whole IgG ITs. Moreover, the chemical linkage allows a great range of available toxins to choose from.

Recombinant fusion toxins

Recombinant fusion toxins are prepared by genetically splicing a toxin encoding DNA to a gene encoding for a MoAb-fragment or other cell binding ligand, and expressing the resulting construct as a single chain fusion protein. This approach is especially suited for the truncated bacterial toxins, which contain a native protease-sensitive sequence required for intracellular dissociation. Plant toxin-based ITs, in contrast, require post-translational removal of the interchain peptide sequence to facilitate intracellular ligand dissociation. Ricin A-based fusion toxins compromising an artificial protease sensitive linker, have thus far demonstrated only modest cytotoxicity.[201]

MoAb-based recombinant immunotoxins typically contain monovalent scFv or dsFv fragments (see Figure 2). This is not only for technical reasons (bacterial expression systems not allowing expression of whole IgG), but also since the small size is considered an important advantage for the penetration of solid tumors.[202,203] Some potential disadvantages should be noted as well. The absence of IgG Fc-parts prevents potentially beneficial interactions with the patient's immune system (see Paragraph 1.5). Moreover, the smaller size results in an accelerated clearance that, together with the monovalent binding, may negatively affect tumor saturation. The limited choice of toxins may also be a disadvantage, as the intracellular routing can be suboptimal for the available toxins. Another concern is that the reduced size may result in an increased renal excretion associated with profound renal toxicity.[204]

The ongoing developments in genetic engineering are likely to solve most of these problems. An example forms the fusion, by various techniques, of Fv fragments into di- or multivalent molecules.[205] Depending on the Fv fragments applied, these can be mono, bi or multi-specific. The resulting molecules often demonstrate improved pharmacokinetics and have affinities equalizing or surpassing that of whole IgG. The additional incorporation of constant parts of the IgG heavy chain results in so called minibodies possessing natural IgG effector functions. The phage display technology is another recent development having a major impact on the production of recombinant ITs. The expression of scFv or Fab' fragments on the surface of filamentous phage enables the isolation of specific MoAb fragments from huge phage-display libraries, thus circumventing the conventional immunization and hybridoma technology. Libraries derived from human donor tissue (*e.g.* PBLs, spleen, tonsil or bone marrow) provide fully human antibody fragments of the desired specificity. These fragments can readily be engineered into the preferred form and spliced to an appropriate toxin-encoding gene. Considering the rapid developments within this area, recombinant production of ITs will undoubtedly become the future standard, surpassing conventional biochemical techniques in rapidly generating homogeneous batches with great flexibility regarding the form of ligand and toxin. Moreover, the greatest promise is still to be fulfilled, the generation of entirely human constructs compromising a human ligand fused to a human enzyme equalizing the conventional toxins in efficacy.

1.5 NON-TOXIN-BASED EFFECTOR MECHANISMS

Apart from its role as toxin transporter, the MoAb moiety contains intrinsic effector mechanisms that may significantly contribute to the overall therapeutic efficacy. These include the following:

The MoAb Fc-part

The MoAb Fc-part may fix complement C1q or bind to Fc receptors expressed on various immune cells.[206] Triggering the complement cascade results in direct complement-dependent cytotoxicity (CDC), as well as in the activation of several immune effector cells. Binding of Fc receptors initiates a diversity of biological responses as well, including antibody dependent cellular cytotoxicity (ADCC), phagocytosis, endocytosis, release of inflammatory mediators, and enhancement of antigen presentation.

Though the potential anti-tumor effect of these mechanisms has been clearly demonstrated in xenografted tumor mouse models, the full clinical relevance has to be determined yet.[207-209] Several studies report of complement deposition or mononuclear infiltrates in post-treatment biopsies of cancer patients treated with unconjugated (murine) MoAbs.[210,211] In a comparative clinical trial of two CAMPATH (CDw52) rat isotype-switch variants, the most effective target cell depletion was observed with the

ADCC-inducing IgG2b MoAb.[212] Moreover, CDC and ADCC are both considered *in vivo* effector mechanisms of chimeric anti-CD20 MoAbs in the treatment of B cell malignancies.[213-218]

Interference with cellular functions

The binding of the MoAb itself may interfere with cellular functions. The MoAb may block or mimic the binding of natural ligand. This may directly induce growth arrest or apoptosis, or the cell may be deprived of a critical survival/proliferation signal.

Members of the type I growth factor receptor family, including epidermal growth factor (EFG) receptor (EGFR, erbB1) and HER-2 (erbB2), have been implicated in the cellular transformation of several human malignancies.[219-223] The antiproliferative effect of EGFR and HER-2 blocking MoAbs has been demonstrated in several preclinical studies, including xenografted mouse tumor models.[224-228] Moreover, a humanized version of a HER-2 blocking MoAb induced objective clinical responses in patients with breast cancer.[13] Currently, combinations of anti-EGFR and anti-HER-2 MoAbs with other therapeutic agents are clinically evaluated as accumulating data suggest these MoAbs enhance the response to certain chemo- and hormone therapies.[229-231]

Humanized anti-CD20 MoAb Rituximab forms an example of a therapeutic MoAb which clinical efficacy might partly be mediated by apoptosis induction. CD20 is a signaling protein involved in the regulation of B-cell proliferation, and is expressed on nearly all normal and malignant mature B cells. Ligation of CD20 by several MoAbs induces cell death by apoptosis.[232-236] In addition to ADCC and CDC, this may contribute to the impressive responses observed in the treatment of CD20-positive neoplasms with unconjugated CD20 MoAbs.[237-240]

Anti-idiotype responses

The MoAb's immunogenicity is a major obstacle potentially preventing multiple cycles of therapy. The reverse side is that the humoral responses mounted by the patient may elicit host anti-target cell antibodies as well. According to the 'idiotypic-network', the administered MoAb (Ab1) may induce host anti-idiotype antibodies (Ab2), which in turn may provoke host anti-anti-idiotype antibodies (Ab3), which in part bind the antigen of the Ab1 MoAb.[241] Indirect support for this concept was provided by the treatment of colon carcinoma with murine MoAb 17-1A. The presence of Ab3 antibodies appeared to be a positive prognostic factor for prolonged survival.[242-248] Other studies have been entirely based on exploiting the anti-idiotype strategy. In these studies, patients were administered Ab2 antibodies, mounted in animals immunized with a tumor-specific Ab1 MoAb (e.g. CO17-1A, HMW-MAA, CEA). Together with the formation of Ab3, several clinical responses could be observed, providing the proof of principle.[249-254]

Relevance of MoAb-based effector mechanisms for IT efficacy

There are several reports which confirm that the above effector mechanisms may contribute to the IT efficacy as well.

Hertler *et al.*, for instance, observed that an anti-CD5 IT (T101-ricin A) induced a rapid but transient fall in white blood cells of patients with chronic lymphocytic leukemia.[255] This response was attributed to an interaction of the MoAb-moiety with the reticuloendothelial system.[143,255] Yokota *et al.* demonstrated in a mouse tumor-xenograft model that the interaction with macrophages enhanced the anti-tumor efficacy of an IT-combination.[42,256] More recently, Flavell *et al* demonstrated in a mouse model that part of the anti-leukemia effect of a CD7-saporin IT was delivered by ADCC through recruitment of NK cells.[257] Vitteta *et al.* provided an example of the MoAb-binding contributing to the IT efficacy. They demonstrated that the cytotoxicity of an anti-CD19 IT was partly based on the growth inhibitory signal induced by binding of the CD19 antigen.[258,259] This binding also impaires the activity of the drug efflux pump P-glycoprotein (P-gp), leaving target cells more susceptible for chemotherapy.[260] For anti-CD3 ITs, it has been demonstrated that binding of the CD3/T cell receptor complex (CD3/TCR) attributes to the IT efficacy by inducing activation induced cell death (AICD).[261-263] Moreover, when treating immune disorder, the binding of the CD3/TCR may block antigen stimulation.[263] Similarly, targeting activated T-cells with anti-CD25 ITs may decrease ongoing activation by blocking the IL-2 signaling.

1.6 LESSONS FROM CLINICAL TRIALS

A variety of ITs has been tested in the clinic for diverse indications. Both recombinant and chemical conjugated ITs have been tested, administered by bolus as well as continuous infusion schedules. Most of these studies have been Phase I/II trials, designed to determine the dose limiting toxicity, maximal tolerable dose, and to gather information regarding biological and clinical efficacy. Due to the nature of these studies, it is too early to draw definite conclusions regarding the administration schedule or type of IT which are eventually to be preferred for *in vivo* use. Nevertheless, some interesting trends have been observed allowing the following general conclusions.

The observed toxicities can roughly be divided in 'general toxicities' directly mediated by the toxin moiety, and 'specific toxicities' due to binding of the cell targeting ligand to bystander tissue. An example of a general toxicity is vascular leak syndrome (VLS), characterized by hypoalbuminaemia, weight gain, peripheral oedema, pleural effusion and, occasionally, life threatening pulmonary oedema. Though observed with all applied bacterial and plant toxins, dose limiting severe VLS appears to be predominantly associated with ricin (A)-based ITs. Generally, VLS is transient and ceases shortly after discontinuation of IT administration. The second dose limiting toxicity reported for dgA-based

ITs is myalgia, rarely associated with rhabdomyolysis. Reversible elevations of serum liver enzymes have frequently been reported for ITs constructed with DT, PE, type I RIP, and blocked ricin.[264-266] Hepatotoxicity has not been associated with dgA-ITs.

An example of specific toxicity has been reported by Pai *et al*. They observed an unexpected and severe central nervous system toxicity when clinically evaluating an IT (OVB3-PE) reactive with human ovarian cancer. The underlying cross-reactivity with normal human brain tissue was not detected during preclinical screening. Similarly, an IT reactive with breast cancer (260F9-RTA) caused severe sensorimotor neuropathy due to unexpected reactivity with Schwann cells. To predict and to anticipate on specific toxicities, ITs are to be extensively screened pre-clinically using a broad panel of human tissues from multiple donors. Nevertheless, MoAb-antigen interactions might be missed due to tissue fixation altering the antigenicity or destroying certain epitopes. The same holds true for the opposite, epitopes may be exposed that are hidden, or only marginally accessible, under normal physical conditions (*e.g.* intracellular antigens or antigens protected by the blood-brain barrier). Cross-reactivity observed *in vitro* should, therefore, not automatically eliminate a MoAb from consideration.[27,267] In general, cross-reactivity observed during preclinical screening asks for additional studies in relevant test animals.

A common finding among IT trials is the occurrence of humoral responses to either the cell targeting ligand or the toxin component. For patients with hematological disorders the incidence of humoral anti-IT responses is typically in the order of 20-30%.[99,263,268-273] The immunogenicity rate seen in ITs trials against solid tumors, in contrast, is often 90 to 100%.[21,29] Many of these responses can be detected within 10 days of the first administration. Though hypersensitivity reactions are rare, repeated dosages may be less effective due to accelerated plasma clearance or the formation of inactive immune-complexes. Notably, extensive clinical responses have been seen even in the face of anti-IT antibodies.[25,274,275]

The accessibility of target cells is a major factor determining the IT-efficacy. So far, solid tumors are treated with only limited success. This may not only be due to heterogeneous blood supply, but also to tight junctions between tumor cells and a high interstitial pressure. Though tumor penetration of small recombinant toxins is more effective, this appears to be counterbalanced by their rapid clearance by the kidney. The $T_{1/2}$ of the recombinant conjugates is often less than 1 hour, as compared to 6 or more hours for whole IgG-containing ITs. The treatment of diffuse hematological and immunological disorders has proved more successful, with overall clinical response rates ranging from 12-75%.[276] Though many of these responses were partial and often transient, this is not such a bad score considering the early nature of most studies (mostly Phase I or II trials). So far, one immunotoxin has actually passed the registration process. The FDA approved recombinant toxin DAB389IL2 (Ontak) for the treat-

ment of patients with advanced or recurrent cutaneous T-cell lymphoma.

Though responses have been obtained at safe dose levels, some important issues have to be addressed in order for ITs to become broadly effective therapeutics. Major challenges include the prevention of severe toxin-induced vascular leakage, the reduction of immunogenicity, and improving on the penetration of solid tumors. The optimal clinical benefit is likely to be obtained in an adjuvant setting, following conventional 'target-cell-debulking' therapy. Simultaneous application of multiple ITs may be necessary to solve the problem of heterogeneous antigen expression.

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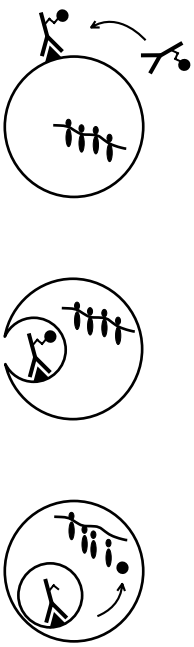
CHAPTER 2

IMMUNOTOXINS IN HEMATOLOGY



CONTENTS

2.1 HEMATOPOIETIC STEM CELL TRANSPLANTATION:

The transplantation of hematopoietic stem cells (SCT) forms a world-wide accepted method for the reconstitution of normal hematopoiesis in patients treated for a hematological malignancy or otherwise suffering from a defective hematopoietic or immunologic system. The stem cells can be derived from the patient himself (autologous transplantation) or, from a suitable donor (allogeneic transplantation). Major obstacles for successful outcome of SCT form recurrence of the malignancy and, in case of allogeneic SCT, graft-rejection and graft-versus-host disease (GVHD). ITs have been used in an attempt to improve on the outcome of transplantation by specifically removing undesirable cell types from either the stem cells graft or the patient. These can be malignant cells as well as over-reactive or misdirected immunocompetent cells (schematically depicted in Figure 1). Paragraph 2.2 and 2.3 review the current status of the SCT-related clinical applications of ITs described so far.

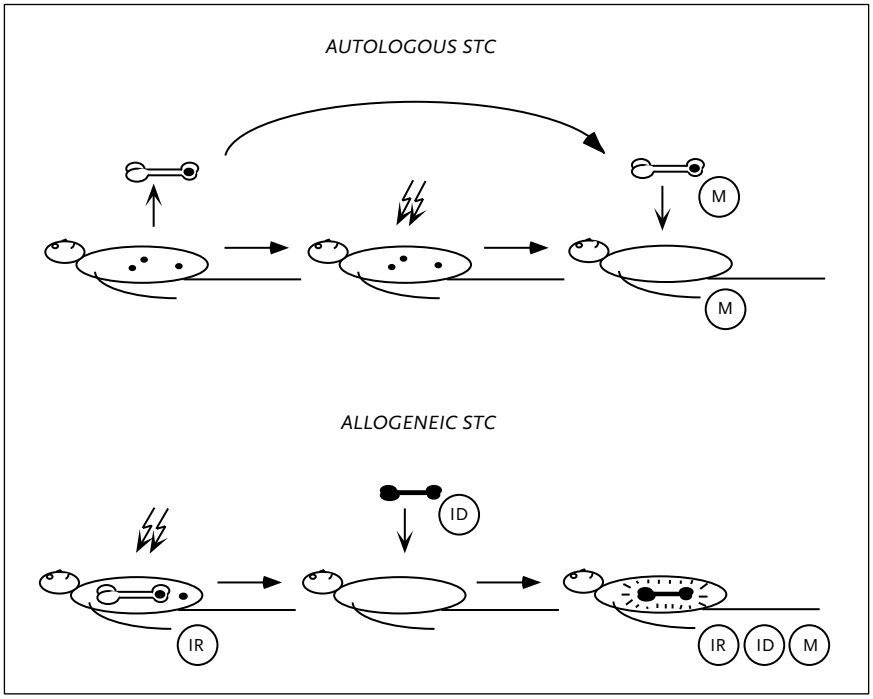


Figure 1 *Schematic representation of the stem cell transplantation (STC) procedure*

A patient suffering from a hematological malignancy is treated with extensive radio and/or chemotherapy for the elimination of malignant cells. In order to restore the affected hematopoiesis the patient is then transplanted with hematopoietic stem cells previously collected during clinical remission (autologous STC), or derived from a suitable donor (allogeneic STC). Adverse events negatively affecting the outcome of SCT are recurrence of the malignancy, and, in case of allogeneic STC, rejection of the stem cell graft and graft-versus-host disease (GVHD). With their specific toxicity, ITs can be used for the ex vivo or in vivo elimination of over-reactive or misdirected immunocompetent cells (either of donor or recipient origin: indicated as ID and IR, respectively), or residual malignant cells (indicated as M).

2.2 EX VIVO PURGING OF STEM CELL GRAFTS

Allogeneic stem cell grafts

Immunocompetent T cells in the graft play a key role in allogeneic SCT. Their (minimal) presence is required for a successful engraftment. Besides, they may contribute to the so-called graft-versus-leukemia effect (GVL), which involves the elimination of residual malignant cells. Unfortunately, they may also initiate graft-versus-host disease (GVHD), a major cause of morbidity and mortality after allogeneic STC. The partial depletion of T cells from allogeneic marrow grafts has been the first reported clinical use of ITs. The initial studies used ricin A-chain or ricin holotoxin conjugated to MoAbs against CD5,

CD3 or a mixture of CD3, CD5 and CD11a.[1-4] The *ex vivo* application allowed the addition of cytotoxicity enhancer NH_4Cl (ricin A-chain) or an excess of lactose to prevent aspecific binding (ricin holotoxin). The T-cell depletion ranged from 1-3 logs for the individual ITs, to up to 4 logs for the combination. All studies noted a significant decrease in the incidence of severe GVHD. Unfortunately, the removal of T cells appeared to be a double-edged sword, associated with poor engraftment and an increased incidence of relapse of the underlying disease. The same holds true for other immunological or physical methods used for (partial) T cell depletion or positive selection of CD34 positive cells.[5-7] Even though some of these methods enable the exact control of the number of T cells in the final graft, SCT remains a complicated procedure, often balancing between graft-failure and leukemic relapse on one side and life-threatening GVHD at the other. Ideally, future purging techniques should deplete only those T cells destined to participate in the anti-host reaction, while conserving those with anti-leukemia reactivity. A step in that direction is the ongoing identification of dominant GVHD-associated CTL epitopes.[8] In combination with the tetrameric HLA-peptide complexes technology ('tetramers'), this might bring back a role for ITs. Conjugating toxins to TCR-specific tetramers, may create highly selective ITs capable of killing CTL with undesired specificities.[9,10] Alteratively, T-cell antigens might directly be fused to a toxin, as has been demonstrated for myelin basic protein.[11] The final success of these strategies is dependent on the degree to which GVHD and GVL can be separated.

Autologous stem cell grafts

Autologous SCT forms an alternative when no suitable donor is available. In this case, the stem cell graft is collected during remission and prior to the destruction of bone marrow by chemo- and/or radiotherapy. This scenario introduces the risk of persistent malignant cells within the graft contributing to a relapse upon re-infusion. ITs has been used for the purging of malignant cells from autologous grafts of patients with T-lineage acute lymphoblastic leukemia or T cell lymphoma. Though these studies demonstrated no adverse effect on engraftment, a clear clinical benefit regarding relapse free survival was hard to prove.[4,12,13] A conclusive statistical evaluation will require large randomized clinical trials. Nevertheless, genetic marking studies clearly have demonstrated the contribution of 'graft-originating' malignant cells to the relapse following autologous SCT for neuroblastoma and CML.[14,15] These results still argue for maximal tumor reduction of autologous grafts, *e.g.* by the IT-based purging of positively selected CD34-positive hematopoietic progenitor cells.[16,17]

2.3 TREATMENT OF GVHD AND STEM CELL REJECTION

Acute GVHD (aGVHD) and accompanying immunosuppression continues to be the major cause of morbidity and mortality in patients receiving allogeneic SCT.[18] With the

standard prophylaxis (typically cyclosporine, often combined with methothrexate) GVHD occurs in 30-70% of HLA-matched recipients and contributes to death in 20-40% of those affected. Even if a patient survives severe GVHD, this generally results in long-lasting disability and morbidity leading to repeated admission to hospital.

The etiology of aGVHD is considered a multi-step process, already initiated by the preparative treatment of the host with chemo- and/or radiotherapy (Figure 2).[19,20] The

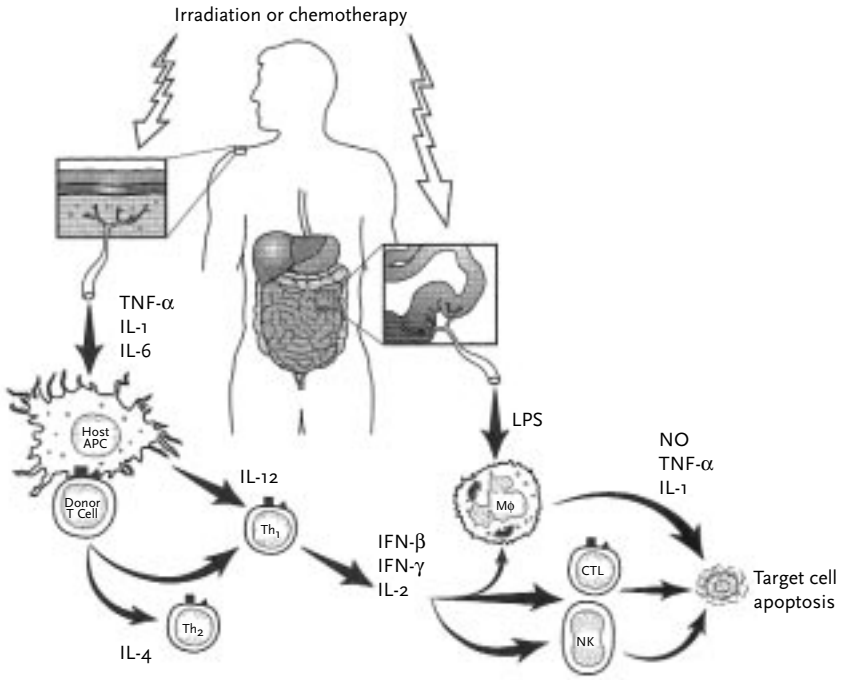


Figure 2 *Schematic representation of the immunopathophysiology of GVHD*

GVHD is considered a multi-step process. The pretransplant conditioning regimen induces damage of host tissues resulting in the secretion of inflammatory cytokines (TNF-α and IL-1) and the subsequent upregulation of MHC antigens. These conditions favor the interaction with allo-reactive donor T cells, which upon activation secrete IL-2, INF-γ. These cytokines induce further T-cell expansion and activate monocytes, macrophages and NK-cells to produce IL-1, TNF-α and IL-6. Altogether, this cascade may cause serious direct and indirect cytotoxicity to epithelial cells of skin, liver and gut.
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inevitable damage to the gastrointestinal tract, liver, and blood vessels induces an inflammatory reaction associated with the release of IL-1 and TNF-α and the over-expression of cytoadhesion molecules and MHC class II antigens. These events lead to an enhanced interaction of allo-reactive donor-T cells with major or minor HLA antigens on the affected tissues. Activation of these cells results in the production of

cytokines (IL-2 and INF-γ) which in turn activate additional effector cells, like monocytes, macrophages and NK-cells, to produce inflammatory proteins (IL-1, TNF-α and IL-6). Altogether, this may result in serious direct and indirect cytotoxicity to epithelial cells of skin, liver and gut.

When prophylaxis has failed, severe aGVHD is usually treated with low dose corticosteroids. If the reaction progresses, the dose may be increased or, alternatively, polyclonal antithymocyte/antilymphocyte globulin (ATG/ALG) or experimental immunosupressive agents may be applied. Most of the experimental therapies are focussed on the removal of T cells or interference with their IL-2 signaling. Based on their capacity of eliminating specific cell populations, IT may also be suited for this type of therapy.

H65-RTA, a ricin A-based anti-CD5 IT, is the first IT clinically used for the *in vivo* treatment of aGVHD. In the initial studies, the use of H65-RTA resulted in extensive responses in about half of the patients with steroid-resistant aGVHD (~25% complete responses).[21-23] Compared to historical ATG-treated controls, the overall responses rates obtained with H65-RTA were higher (52 vs. 31%) and the median survival was longer (148 vs. 80 days).[23] Unfortenately, in later comparative trials H65-RTA was not more effective than high-dose corticosteroids or ATG.[24,25] Additionally, the use of H65-RTA for the prophylaxis of aGVHD or stem cell rejection revealed no clear advantage over current cyclosporin-based regimens.[26-30]

Several animal studies now suggest that CD3-directed ITs may be more suitable for the control of GVHD or graft-rejection than their anti-CD5 counterparts. The group of Vallera and Blazer studied the therapeutic potential of a hamster derived anti-murine-CD3 MoAb in an aggressive murine model of established GVHD. Conjugated to ricin A, the CD3 MoAb appeared to be far more effective than a similar ITs directed against the murine CD5-equivalent (Lyt-1), despite both ITs having similar clearance and biodistribution.[31] Its efficacy could partly be explained by the CD3 MoAb inducing modulation of the T-cell receptor and T cell depletion.[32] Moreover, conjugation to ricin A dramatically improved the depletion efficacy of both the IgG and $F(ab')_2$-fragments.[31,33] Vallera *et al*. are now trying to identify the most appropriate form of anti-CD3 ITs for clinical use. Their latest report addressed the evaluation of a single chain fusion toxin, comprising the Fv fragment of the anti-murine-CD3 MoAb genetically linked to truncated diphteria toxin.[34-36] Due to its relatively small size (68 kDA), this fusion toxins was readily filtrated into kidney, causing severe and irreversible renal damage. This problem appears now to be solved by the introduction of a cysteine residue at the c-terminus of the sFv part, resulting in the formation of disulfide bonded bivalent IT with an expanded therapeutic window.[36]

Neville *et al*. studied the T-cell depletion efficacy of an anti-rhesus-CD3 murine IgG1 MoAb conjugated to a diphteria toxin binding mutant (FN18-CRM9).[37] This construct was able of depleting >99% of T cells from both the blood and lymph node compart-

ments of healthy rhesus monkeys. Restoration of the T-cell compartment took 12 to 30 days and appeared to be thymus dependent. Depletion activity of native FN18 was limited to the blood compartment and lasted only for a day.[37] Additional primate studies focussed on the use of FN18-CRM9 in protocols aiming at tolerance induction. Encouraging results were obtained, including the induction of long-term tolerance in kidney and pancreatic islet transplant recipients.[38-44] As an extension to these primate studies, an anti-porcine CD3-IT has been generated (pCD3-CRM9) for evaluation in a non-myeloblative stem cell transplantation model established in miniature swine.[45] When pCD3-CRM9 was added to a mild preparative regimen, infusion of blood stem cells from leucocyte antigen-matched animals resulted in stable mixed chimerism without aGVHD occuring.[46] Moreover, this protocol provided donor-specific tolerance as indicated by long-term acceptance of donor skin and consistent rejection of third party skin.

2.4 TREATMENT OF LEUKEMIAS AND LYMPHOMAS

Their diffuse nature, and the relative abundance of tissue-specific antigens, make hematological malignancies an attractive target for IT therapy. Table 1 summarizes the outcome of the major studies reported so far. Most of them are directed at B cell derived malignancies, mainly due to their high incidence as compared to T-cell neoplasms (ratio of about 5:1).

Vitetta and colleagues performed a series of studies in which they evaluated RFB4-dgA (anti-CD22) and HD37-dgA (anti-CD19) for the treatment of relapsed Non-Hodgkin's Lymphoma (NHL). Initial studies with RFB4-dgA demonstrated no particular advantages of Fab'-ITs over whole IgG-ITs with regard to clinical responses, toxicities or immunogenicity. Subsequent studies were performed with the more cost-effective whole-IgG ITs. Both RFB4-dgA and HD37-dgA were administered as bolus and continuous infusions, revealing no obvious advantage of one schedule above the other, and resulting in a comparable maximal tolerable dose (MTD: ~16-20 mg/m^2/8 days for both ITs). The most prominent toxicities were VLS, myalgia, rhabdomyolysis and aphasia. Overall response rates of RFB4-dgA were higher than those obtained with HD37-dgA (~25% and 10%, respectively). Based on the synergistic effect observed in preclinical studies[47], the combination of HD37-dgA and RFB4-dgA (Combotox) is now being clinically evaluated.[48] The first study appears to be hampered by unpredictable clinical courses, attributable to batch-dependent multimerization of HD37-dgA. Especially patients lacking circulating tumor cells were vulnerable for severe VLS and hemolytic uremic syndrome (HUS). Three deaths were noted, of which two were probably IT-related. Further studies are planned using non-aggregate-forming formulations of HD37-dgA.

Another comprehensive series of clinical studies is performed with B4-bR, an anti-CD19

Table 1 *Major clinical trials of IT for the treatment of hematological malignancies*

	Antigen	Disease[a]	Administration	#Patients
T101-RTA	CD5	B-CLL	b.i. twice wkly x 4 wk	9
H65-RTA	CD5	CTCL	b.i. daily x 10 d	14
Anti-CD7-dgA (DA7)	CD7	T-NHL, T-LGL	b.i. daily x 5 d	11
HD37-dgA	CD19	B-NHL	b.i. every 48h x 4 c.i. for 8 d b.i. every 4h x 4	23 10 8
B43-PAP	CD19	B-ALL	c.i. for 5 d, 1 or 3 cycles	17
RFB4-dgA (Fab')	CD22	B-NHL	b.i. every 48h x 2-6	15
RFB4-dgA (IgG)	CD22	B-NHL	b.i. every 48h x 2-12 c.i. for 8 d	26 18
RFB4-dgA (IgG) & HD37-dgA	CD19/CD22	B-NHL	c.i. for 8 d	22
B4-bR	CD22	B-cell neoplasms B-cell neoplasms B-NHL, postABMT, Ph I B-NHL, postABMT, Ph II B-NHL, postABMT, Ph III AIDS-NHL, relapsed AIDS-NHL, untreated	b.i. daily x 5 d c.i. for 7 d c.i. for 7 d c.i. for 7 d, every 14 d c.i. for 7 d, every 7 d c.i. for 28 d c.i. for 7 d	25 34 12 49 157 9 28
RFT5-dgA	CD25	HD	b.i. every 48h x 4, 1-4 cycles	25
Anti-Tac(Fv)-PE38 (LMB-2)	CD25	B & T lymph/leukemia, HD	b.i. every 48h x 3	35
BerH2-SAP	CD30	Hodgkin's	b.i. on day 1 ± 7	12
DAB486IL-2	IL-2R	NHL, Hodgkin's, CLL NHL, Hodgkin's, CLL NHL CTCL & Sezary	b.i. on day 1, 3-5, 14-20 b.i. daily x 5 d b.i. daily x 5 d b.i. dly x 5 d, every 21 d	18 15 23 14
DAB389IL-2	IL-2R	Mycosis fungoides	b.i. dly x 5 d every 21 d	71

MTD	Toxicities[b]	Human anti-IT Ab	Responses[c]	Ref.
24-112 mg/m² total	Fever, nausea, rash	1/9	Rapid transient fall in WBC	68,69
0.33 mg/kg/d	VLS, fever, chills, nausea, fatigue	10/14	4 PR	70
0.2 mg/kg/d	VLS, aphasia	1/11	0 CR, 2 PR	71
16 mg/m²/8 d	VLS, rhabdomyolysis	5/15	1 CR, 1 PR	72
19.2 mg/m²/8 d	VLS, acrocyanosis	2/8	1 PR	72
8 mg/m²	VLS, hypotension, rhabdomyolysis	2/7	1 PR	73
1.25 mg/kg	VLS, myalgia	0	4 CR, 1 PR	74
75 mg/m²	VLS, myalgias, aphasia, rhabdomyolysis	4/14	5 PR	75
26-40 mg/m² total	VLS, myalgias, aphasia, rhabdomyolysis	9/24	1 CR, 5 PR	76
19.2 mg/m²/8 d	VLS, aphasia, hypotension	6/14	4 PR	77
≤10 mg - 30 mg/m²	VLS, HUS	6/22	2 PR	48
250 μg/kg total	AST, ALT, PLTs, VLS, fatigue	9/25	1 CR, 2 PR	78
350 μg/kg total	AST, ALT, PLTs, VLS, myalgia, edema	24/34	2 CR, 3 PR	79
280 μg/kg total	AST, ALT, PLTs, VLS, fever	7/12	7 relapse free, MFU 4 years	80
210 μg/kg total	AST, ALT, PLTs, VLS	23/49	27 relapse free, MFU 38 months	50
210 μg/kg total	PLTs, VLS, fatigue, dyspnea	NA	Comparable with control	81
560 μg/kg total	AST, ALT	3/9	1 CR, 1 PR	82
140 μg/kg total	AST, ALT, fever, myalgia, fatigue	8/28	14 CR, 12 PR	83
15 mg/m²/cycle	VLS, myalgias, hypersensitivity	7/15	2 PR	84,85
120 μg/kg total	AST, ALT, cardiomyopathy, diarrhea, fever	11/35	1 CR, 7 PR	86
0.4 mg/kg/d	AST, ALT, PLTs, VLS, fever, fatigue	12/12	8 PR	87,88
0.1 mg/kg/d	AST, ALT, fever, dyspnea, rash	7/18	1CR, 2 PR	51
0.2 mg/kg/d	AST, ALT, hypoalbuminemia, hypersensitivity	6/14	1 CR	89
0.3 mg/kg/d	PLTs, r]enal insufficiency, hemolysis	15/20	2 PR	52
0.2 mg/kg/d	AST, ALT	8/14	1 PR	55
9 or 18 μg/kg/d	AST, ALT, VLS, hypersensitivity	70/71	3 CR, 18 PR	90,91

[a] *B-CLL, B-cell chronic lymphocytic leukemia; CTCL, cutaneous T-cell lymphoma; T-NHL, T-cell non-Hodgkin's lymphoma; T-LGL, T-cell large granular lymphocyte leukemia; B-NHL, B-cell non-Hodgkin's lymphoma; B-ALL, B-cell acute lymphoblastic leukemia; postABMT, post autologous bone marrow transplantation; AIDS-NHL, AIDS-related non-Hodgkin's lymphoma; HD, Hodgkin's disease; AML, acute myeloid leukemia.*

[b] *VLS, vascular leak syndrome; HUS, hemolytic uremic syndrome; AST, aspartate amino transferase; ALT, amino alanine transferase; PLTs, thrombocytopenia*

[c] *WBC, white blood cell counts; PR, partial response; CR, complete response; MFU, median follow up.*

MoAb conjugated to blocked ricin (B4-bR). Following the evaluation of various dosing protocols and different clinical settings, B4-bR was the first IT to be tested in the minimal disease setting. B4-bR has been administered to patients with NHL brought in complete remission with high-dose chemotherapy and stem cell transplantation. Regrettably, no beneficial effects on disease free survival could be demonstrated in a randomized Phase III trial.[49,50] The hepatotoxicity of bR was and important factor limiting therapeutic success by reducing the therapeutic window.

Of note is the good performance of IL-2 receptor (IL-2R) targeted recombinant ITs in certain hematological diseases. Truncated diphtheria toxin spliced to IL-2 (DAB486IL-2 and the improved version DAB389IL-2) showed impressive efficacy in treating CD25-positive refractory cutaneous T-cell lyphoma (CTCL), with overall response rates of approximately 30%.[51-58] DAB389IL-2 (Ontak) has been approved by the FDA (May 1999) for the treatment of patients with persistent or recurrent CD25-positive CTCL.

The group of Pastan created an alternative anti-IL-2R IT by genetically linking an anti-CD25 MoAb Fv-fragment to truncated Pseudomonas exotoxin (LMB-2).[59-61] This IT differs from DAB389IL-2 in that it binds CD25 (IL-2R α-chain) with high affinity also in the absence of the IL-2R β and γ chain. This may be an advantage for malignancies solely expressing the IL-2R α-chain, like the Reed Sternberg cells in Hodgkin's disease. LMB-2 has been adminstered to patients with various CD25-postive hematological malignancies. Remarkably, all four patients with hairy cell leukemia (HCL) responded to therapy, with one complete responder of more than 20 months of duration. Additionally, four partial responses were observed in CTCL (n = 1), CLL (n = 8), HD (n = 11), and adult T-cell leukemia (n = 2). Additional *in vitro* studies with freshly derived patient cells confirmed the particular vulnerability of HCL cells as compared to B-CLL cells expressing equal or higher numbers of CD25 per cell.[62]

The above examples represent a proof of principal that IT-based therapy of hematological malignancies can be clinically useful. Several clinical responses could be noted in refractory patients with bulky disease. The ongoing and coming clinical trials will be crucial in determining the future role of ITs. Not only will they teach us which particular hematological diseases are vulnerable for what type of IT, they also should identify the optimal clinical setting for maximal therapeutic benefit.

2.5 STARTING POINT OF THESIS

The UMC St Radboud was among the first to introduce the IT research in the Netherlands, in 1983. This research was initially focused on identifying the factors influencing the activity of IT, like the nature and density of target-antigens, the internalization rate, the use of toxicity enhancers, and the activation state of the target cells.[63-66] These stud-

ies resulted in the first institutional clinical application, the *ex vivo* purging of autologous marrow grafts of patients suffering from T cell leukemia or lymphoma.[12] Aim of this treatment was to reduce the incidence of a malignant relapse caused by residual malignant cells in the autologous graft. The more or less routinely *ex vivo* purging of bone marrow can be regarded as the starting point of this theses. As such, this application initiated additional research focussing at ways to improve the purging efficacy and to reliably monitor its efficacy in a heterogeneous population such as bone marrow grafts. At the mean time, the international excitement regarding the therapeutic possibilities of the 'magic-bullit concept' more or less drove the IT from the laboratory to the clinic. Several institutes, mostly US academic centers, initiated phase I/II studies based on the IT-targeting of diffuse and solid tumors and varying immune diseases. Encouraged by the outcome of these early studies, especially regarding the treatment of diffuse disorders, and motivated by the urgent clinical need, the Department of Hematology decided to develop an IT-based therapy for lifethreatening aGVHD. Additionally, another research line was started studying the anti-tumor activity of an anti-B cell IT in a mouse xenografted tumor model. The next part of this thesis forms a selection of the institutional IT-research during the last decade. It covers part of the early *in vitro* work (Chapter 3 to 5), and the set up and preliminary results of a clinical pilot study for the treatment of aGVHD (Chapters 6 and 7). Notably, the complementary work, addressing the IT-based treatment of B cell malignancies, is described in the thesis of Van Horssen.[67]

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CHAPTER 3

CYTOTOXITY OF CD_3-RICIN A-CHAIN IMMUNOTOXINS IN RELATION TO CELLULAR UPTAKE AND DEGRADATION KINETICS

Ypke V.J.M. van Oosterhout

Frank W.M.B. Preijers

Hans M.C. Wessels

Theo de Witte

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ABSTRACT

The cytotoxicity of WT32 (CD3)-ricin A immunotoxin (IT) to the acute lymphoblastic leukemia T-cell line Jurkat was compared with the rate of internalization and intracellular degradation of WT32 and WT32-ricin A during continuous exposure. Moreover the influence of NH_4Cl and monensin on these processes was studied.

Based on protein synthesis inhibition ([^{3}H]leucine incorporation), it appeared that cytotoxicity was not fully expressed directly after exposure to IT due to delay either in the internalization of membrane-bound IT or the action of intracellular ricin A. Varying the duration of incubation and postponing [^{3}H]leucine addition up to 24 h after initiation showed that cytotoxicity occurred in two phases, rapid internalization of initially bound IT followed by a continuous but slower uptake possibly due to reexpression of the CD3 antigen.

No differences were found in the rate of internalization and degradation of ^{125}I-labeled WT32 and WT32-ricin A. Internalization started rapidly after binding at 37°C, was fastest during the first 12 h (~360,000 molecules/cell) and continued for at least 24 h (~420,000 molecules/cell). Exocytosis of intracellularly degraded molecules became measurable after 1 to 2 h of incubation at 37°C and increased up to approximately 400,000 molecules/cell in 24 h. After 4 h of incubation at 37°C the amount of internalized molecules exceeded the amount of WT32 that could maximally bind to the cell membrane (~150,000 molecules/cell) confirming reexpression of antigen.

Addition of NH_4Cl and monensin enhanced the cytotoxicity of WT32-ricin A, probably due to an increased intracellular amount of IT. These agents appeared to reduce strongly the degradation of internalized WT32, resulting in an accumulation of intracellular molecules. NH_4Cl was most effective during the first 12 h of incubation, whereas monensin increased the amount of intracellular WT32 molecules after 2 to 24 h.

Our observations suggest that incubation conditions for the optimal cytotoxicity of IT treatment can be predicted by studying the internalization and degradation of the IT or respective monoclonal antibody.

INTRODUCTION

Monoclonal antibodies (MoAbs) coupled to toxins such as the A-chain of the phytolectin ricin appear to be highly specific ITs for the ex-vivo elimination of both malignant and normal T-cells which occur in autologous and allogeneic bone marrow (BM) grafts, respectively.[1-5] The efficacy of ricin A ITs depends on the number of molecules internalized by target cells and their intracellular routing and processing.[6-11] Ricin A-chain catalytically and irreversibly inhibits protein synthesis by cleaving the N-glycosidic bond of a single adenosine residue of 28S rRNA.[12] Theoretically, a cell can be killed by a single molecule that enters the cytosol[13] but since the bulk of IT is usually transported into cellular compartments where ricin A is either proteolytically cleaved or inactivated by low pH[7,14-16], a sufficient number of molecules must be endocytosed to guarantee cytotoxicity.

Cells with a low density of antigen may survive treatment when the number of internalized IT molecules remains below the required threshold. This problem may be partly overcome by adding lysosomotropic amines (*e.g.*, ammonium chloride, chloroquine) or carboxylic ionophores (*e.g.*, monensin, nigericin) which enhance cytotoxicity by influencing different intracellular processes such as routing and metabolism.[17-21] Alternatively, when the IT is continuously internalized as a result of reexpression of the target antigen, cytotoxic efficacy can be improved by prolonging incubation. However, this may be harmful to normal BM cells, and therefore it is important to determine the optimal period for individual ITs in purging BM grafts.

CD3-ricin A-chain ITs appear good candidates for the selective elimination of malignant and normal T lymphocytes.2,4,22,23 However, optimal incubation conditions remain to be determined. We therefore studied the kinetics of cytotoxicity of WT32-ricin A in the presence or absence of the enhancers ammonium chloride or monensin, and compared these with the intracellular uptake and processing of 125I-WT32 and 125I-WT32-ricin A.

MATERIALS AND METHODS

Cell line

The human acute lymphoblastic leukemia T-cell line Jurkat was cultured in RPMI 1640 (Flow Labs, Irvine, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, Paisley, Scotland), glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator with 5% CO_2 in air at 37°C. Cells were maintained in the log phase.

Monoclonal antibody and immunotoxin preparation

Murine anti-human T-cell MoAb WT32 (IgG2a, CD3)[24] was purified from ascitic fluid by ammonium sulfate precipitation and affinity chromatography with staphyloccocal protein A-sepharose (Pharmacia, Uppsala, Sweden). WT32 was conjugated on a 1 to 1.1 ratio to ricin A using *N*-succinimydyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia) and assayed as described elsewhere.[9] Antibody-binding activity was confirmed by flow cytometry (FCM) and enzyme-linked immunosorbent assay.[9] Nonspecific cytotoxicity was tested on cell lines which did not express the CD3-antigen.[9] Ricin A was kindly provided by Dr. F.K. Jansen (Centre de Recherches Clin Midy, Montpellier, France).

Radioiodination

WT32 and WT32-ricin A were labeled with ^{125}I (Amersham International, UK) using the Chloramine-T method (Hunter, 1973), and then purified by chromatography on a Sephadex G25 column (PD-10; Pharmacia). Eluted fractions containing ^{125}I-WT32 and ^{125}I-WT32-ricin A were pooled, and molecules smaller than M_r 30,000 were removed by means of a centriprep-30 concentrator (Grace B.V., Amicon Division, Rotterdam, Holland). Thereafter radioactivity due to free ^{125}I and fragments smaller than M_r 30,000 was less than 0.3% of the total radioactivity. The concentration of the labeled antibody and its ricin A conjugate was determined by radioimmunoassay, while immunoreactivity was assayed by means of binding limiting quantities of labeled WT32 and WT32-ricin A. Conjugation of ricin A to the antibody was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ^{125}I-labeled IT, followed by autoradiography and scanning densitometry (data not shown).

Cytotoxicity of WT32-ricin A

Culture medium containing 10^6 cells/ml was incubated at 37°C with WT32 ricin A (10^{-8} M) with and without either 6 mM NH_4Cl or freshly prepared 100 nM monensin (Sigma, St. Louis, MO). After 1, 2, 4, 8, 16, and 24 h of incubation, cells were washed with RPMI 1640 containing 1% FCS and then resuspended in fresh medium. They were distributed in triplicate into 96-well microtiter plates (Costar, Cambridge, MA) at a concentration of 10^5 cells/well. Subsequently, cells were immediately incubated with 1 μCi [^{3}H]leucine (Amersham) at 37°C for 1 h, following which they were harvested. The radioactivity was determined, and protein synthesis was expressed as the percentage [^{3}H]leucine incorporation of untreated cells after correcting for non-specific incorporation in the presence of 1 mM cycloheximide.

Cytotoxicity caused by continuous internalization of WT32-ricin A

The increase of cytotoxicity after prolonging incubation might be explained by delayed internalization of IT already bound soon after exposure or slow onset of cytotoxicity. To eliminate differences in cytotoxicity caused by these phenomena rather than by continuous internalization of IT, the assay was modified by introducing an IT-free incubation after cells were washed up to 24 h after the initiation of the experiment (23, 22, 20, 16, 8, and 0 h, respectively), and then proceeding with [^{3}H]leucine incorporation.

Intracellular and surface bound ^{125}I-WT32 and ^{125}I-WT32-ricin A

Following continuous incubation of Jurkat cells with ^{125}I-labeled antibody or its ricin A conjugate, the number of intracellular molecules was determined using a modification of the procedure previously described.[9] Briefly, a suspension of $5x10^5$ target cells was incubated with 10^{-8} M ^{125}I-WT32 or ^{125}I-WT32-ricin A for various times at either 37°C or 4°C at a final volume of 100 µl. Thereafter cells were washed and counted to determine the total amount of associated radioactivity. Subsequently, cells were incubated with isotonic glycine buffer (pH 2) at 4°C for 30 min, to remove surface-bound molecules and then washed, after which radioactivity was again determined. The absolute number of intracellular molecules was calculated from the glycine buffer-treated cells initially incubated at 37°C after correcting for the cells treated with the same buffer at 4°C.

The number of molecules bound to the cell surface was determined by subtracting the amount of intracellular radioactivity from total radioactivity after incubation at 37°C. The maximal number of binding sites was determined by incubation with increasing amounts of ^{125}I-WT32 at 4°C for 1 h.

Degradation of ^{125}I-WT32 and ^{125}I-WT32-ricin A

The degraded and subsequently exocytosed amount of internalized molecules was determined by measuring radioactivity of the trichloroacetic acid (TCA)-soluble fraction of culture supernatant. After incubation with ^{125}I-WT32 or ^{125}I-WT32-ricin A at 37°C or 4°C, cells were pelleted by centrifugation. Seventyfive µl of supernatant was treated with 1 ml of 25% TCA and 75 µl 1% bovine serum albumin (BSA) at 4°C for 15 min and then centrifuged at 1500 G for 15 min. The radioactivity of the soluble fraction from cells incubated at 4°C was substracted from that obtained at 37°C to remove any background radioactivity.

Extracellular degradation was excluded since there was negligible radioactivity found in the TCA-soluble fraction of B-cell line Daudi, which does not express CD3, and of purified surface membranes from disrupted Jurkat cells after incubation with ^{125}I-WT32 or ^{125}I-WT32-ricin A as described by Press *et al*.[19,25] Similar results were obtained after incubating Jurkat with a ^{125}I-labeled control antibody (CD19, IgG2a) or its ricin A conjugate (data not shown), suggesting that no extracellular degradation occurred during incubation.

Internalization ^{125}I-WT32 and ^{125}I-WT32-ricin A

Internalization was defined by the total number of molecules transported across the cell membrane during incubation. The degree of internalization was calculated by adding the fraction which was intracellularly degraded and subsequently exocytosed to the amount of intracellular molecules.

RESULTS

Kinetics of cytotoxicity of WT32-ricin A directly after IT treatment

Prolonging IT treatment led to a reduction in [^{3}H]leucine incorporation from 85% after 1 h to 9% after 24 h Figure 1. This might be explained by continuous binding and internalization of IT. Alternatively it might be the result of slow internalization of IT bound to cells in the first phase of incubation or of a delay in the toxicity of intracellular ricin A molecules.

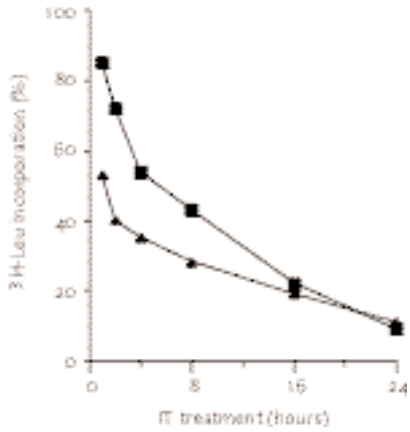

Figure 1
Kinetics of cytotoxicity of WT32-ricin A

Jurkat cells were incubated at 37°C with 10^{-8} M WT32-ricin A for various periods. After IT treatment cells were washed, and directly after (■) or 24 h after (▲) initiation of the experiment they were incubated with [^{3}H]leucine for 1 h. Protein synthesis is expressed as a percentage of [^{3}H]leucine incorporation of untreated cells. Untreated cells incorporated ~20,000 cpm; background incorporation was ~100 cpm. Assays were performed in triplicate; SDs were less than 10%.

Cytotoxicity caused by continuous internalization

Introduction of an IT-free incubation up to 24 h after the initiation of the experiment resulted in an additional decrease of protein synthesis which became less pronounced after prolonged treatment (Fig. 1). The kinetics of protein synthesis measured after the post-IT-treatment period showed a biphasic pattern: a considerable decrease of protein synthesis during the first 2 h of IT treatment (60%), followed by a slower decrease for up to 24 h (89% after 24 h). In this setting the reduction of protein synthesis could only be the result of continuous internalization of IT.

Influence of enhancers of cytotoxicity

The presence of NH_4Cl as well as of monensin resulted in significantly greater decrease of protein synthesis after 1 h (Figure 2), although monensin appeared to be slightly more effective than NH_4Cl (83%, 74% and 47% reduction with monensin, NH_4Cl and without enhancers, respectively). Similar concentrations of NH_4Cl or monensin in the absence of IT were non toxic since [^{3}H]leucine incorporation was unaffected (data not shown). Enhancement of cytotoxicity became less pronounced after prolonging incubation in the presence of IT.

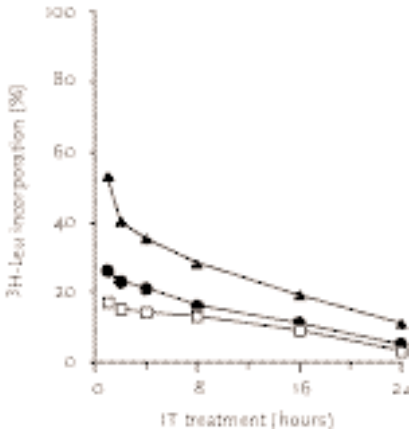

Figure 2
Influence of cytotoxicity enhancers

Jurkat cells were incubated at 37°C with 10^{-8} M WT32-ricin A for various periods in the absence (▲) or presence (●) of 6 mM NH_4Cl or 100 nM monensin (□). After IT treatment cells were washed, followed by an incubation in the absence of IT and enhancers up to 24 h after initiation of the experiment, and then by a 1-h uptake of [^{3}H]leucine. Protein synthesis is expressed as percentage [^{3}H]leucine incorporation of untreated cells. Untreated cells incorporated ~20,000 cpm; background incorporation was ~100 cpm. Assays were performed in triplicate; SDs were less than 10%.

Kinetics of internalization and intracellular degradation of ^{125}I-WT32 and ^{125}I-WT32-ricin A

To confirm the possibility of continuous internalization due to reexpression of antigen as suggested by the pattern of protein synthesis inhibition, the kinetics of internalization and intracellular degradation were studied by means of ^{125}I-labeled WT32 and its ricin A conjugate. The maximal number of binding sites was determined with increasing concentrations of ^{125}I-WT32 at 4°C and was achieved with 4 μg/ml ^{125}I-WT32 (~150,000 molecules) (Figure 3).

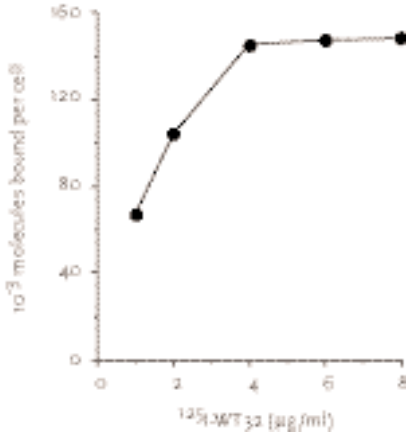

Figure 3
Binding of ^{125}I-WT32 to Jurkat cells at 4°C

Jurkat cells were incubated with 1, 2, 4, 6 and 8 μg/ml ^{125}I-WT32 at 4°C for 1 h. After incubation unbound ^{125}I-WT32 was washed out, and the number of cell-associated ^{125}I-WT32 molecules was determined (●). Assays were perfomed in triplicate; SDs were less than 10%.

Both ^{125}I-WT32 and ^{125}I-WT32-ricin A were internalized at the same rate (Figure 4). At 37°C ^{125}I-labeled antibody and its ricin A conjugate were rapidly internalized during the first 12 h (~360,000 molecules); thereafter internalization continued at a slower rate for up to at least 24 h (between 420,000 and 440,000 molecules). After 4 h the number of internalized molecules surpassed the amount of ^{125}I-WT32 that could maximally bind

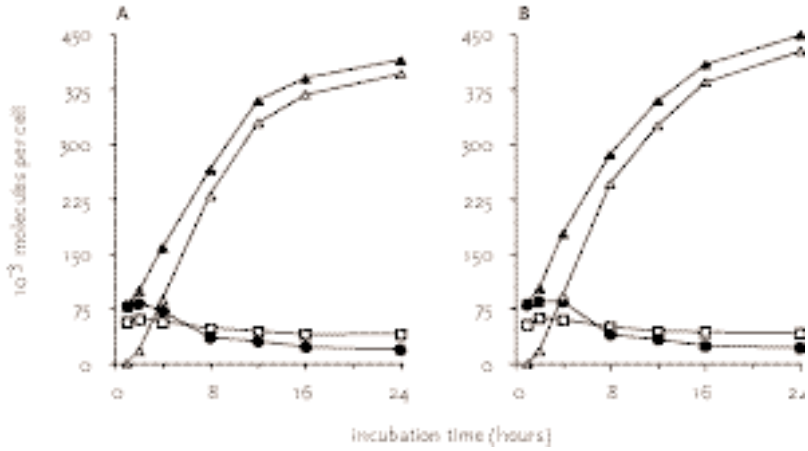

Figure 4 *Internalization and intracellular degradation of WT32 and WT32-ricin A*

Jurkat was incubated at 37°C or 4°C with ^{125}I-labeled WT32 (A) or WT32-ricin A (B) for various periods. Results of cells incubated at 37°C corrected for cells treated at 4°C, as described in "Material and Methods", are depicted. The number of internalized and subsequently degraded and exocytosed molecules was measured by counting TCA-soluble radioactivity of the supernatant of incubation medium (△). The number of surface bound molecules was measured by counting cell bound radioactivity that was removable by glycine buffer pH2 (□). The intracellular number of molecules was measured by counting cell associated radioactivity not-removable by the glycine buffer (●). Absolute amount of internalized molecules was determined as the sum of intracellular molecules and intracellular broken down and subsequently exocytosed molecules (▲). One of three (A) and one of five (B) concordant experiments are shown.

to the cell membrane (~150,000), confirming the continuous reexpression of antigen. The amount of membrane-bound molecules at 37°C (~60,000 after 2 h) decreased slightly during the 24-h period. Binding of ^{125}I-WT32 and ^{125}I-WT32-ricin A at the concentration used was optimal after 2 h at 4°C and remained unchanged up to 24 h (data not shown).

The number of intracellular molecules reached a maximum after a 2-h incubation at 37°C (~80,000 molecules) and declined to about 18.000 molecules after 24 h.

Breakdown and exocytosis of internalized molecules were measurable after 1 h and increased to about 330,000 molecules in 12 h. Thereafter the exocytosis rate was reduced but still continued up to about 400,000 molecules after 24 h.

Influence of cytotoxicity enhancers on the internalization and intracellular degradation

To determine the influence of the cytotoxicity enhancers on the uptake and intracellular degradation of ^{125}I-labeled WT32 and its ricin A conjugate at 37°C, the above-described experiments were repeated with ^{125}I-WT32 in the presence of NH_4Cl (Figure 5A) or monensin (Figure 5B).

The addition of NH_4Cl resulted in a considerable increase in the amount of intracellular ^{125}I-WT32, with a maximum of at least 140,000 molecules between 4 and 8 h. There-

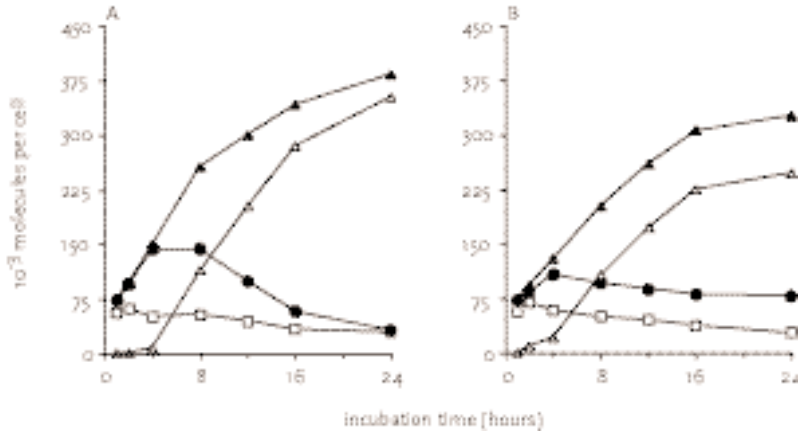

Figure 5 *Internalization and intracellular degradation of WT32*

Jurkat was incubated at 37°C or 4°C with ^{125}I-WT32 for various periods in the presence of 6mM NH_4Cl (A) or 100 nM monensin (B). Results from cells incubated at 37°C corrected for cells treated at 4°C, as described in "Material and Methods", are depicted. Exocytosis of degraded WT32 (△), surface-bound WT32 (□), intracellular WT32 (●) and internalized WT32 (▲) were determined. For additional legends see Fig. 4. One of four (A) and one of three (B) concordant experiments are shown.

after this increase diminished, almost disappearing after 24 h of incubation (Figures 4 and 5A).

Monensin also resulted in an increase of intracellular ^{125}I-WT32, but this was less pronounced during the first 8 h. After 4 h the amount of intracellular ^{125}I-WT32 reached a peak of approximately 100,000 molecules, which gradually declined to 80,000 molecules after 24 h of incubation. However this was larger than the yield either with NH_4Cl or without enhancer after 24 h.

The absolute number of internalized molecules in the presence of NH_4Cl was slightly decreased in comparison to the conditions without enhancers, suggesting that NH_4Cl did not influence the internalization of ^{125}I-WT32 (~380,000 and 410,000 molecules after 24 h, with and without NH_4Cl, respectively). The addition of monensin resulted in a larger reduction in the number of molecules entering the cell (~330,000 molecules after 24 h).

DISCUSSION

The cytotoxicity of WT32-ricin A was not fully expressed directly due to a delay in either the internalization of initially bound IT or the action of intracellular ricin A. This has also been reported by Ravel *et al*.[20] and Sung *et al*.[26], using T101-ricin A (CD5) and 454-ricin A (CD71), respectively. They observed that cytotoxicity increased when measuring protein synthesis was postponed. This phenomenon can strongly influence the outcome of kinetics studies for the determination of the optimal incubation time. To determine the efficacy of prolonging IT-treatment, it is therefore important to standardize the period between initiation of IT incubation and the moment of [^{3}H]leucine exposure. This was assessed by introducing an IT-free incubation up to 24 h after the initiation of the experiment. The reduction of protein synthesis in the current setting showed a biphasic pattern of strong inhibition during the first period followed by a slower inhibition during the second period. This suggests a rapid internalization of initially bound WT32-ricin A during the first period of IT treatment, followed by a more slowly continuous uptake of WT32-ricin A, possibly due to reexpression of CD3 antigen.

In order to confirm the continuous uptake of IT due to reexpression of CD3 antigen, binding, internalization and degradation of WT32 as well as WT32-ricin A were examined after continuous exposure of Jurkat cells to ^{125}I-labeled ligands. Free MoAb and the respective IT were both studied because conjugation of ricin A to the MoAb may affect the internalization or intracellular routing of the complex, depending on the target antigen. It has been reported that conjugation of RFT11 (CD2) to ricin A decreases the intracellular degradation of ligand.[10] In this study we show that there is neither difference in internalization nor in intracellular degradation between WT32 and WT32-ricin A. This has also been described for other MoAbs and ricin A ITs.[6,9,11,27]

Press *et al*.[19] and Geissler *et al*.[18] studied the internalization and degradation of CD3-MoAb ^{125}I-64.1 in T-cells and the T-cell line HPB-ALL after initial pulse labeling of the cell surface with ^{125}I-64.1 at 4°C for 1 h. Washing out unbound ligand prior to incubation at 37°C, however, would reduce the amount that can be maximally internalized due to: (*a*) disturbing the equilibrium between free and bound ligand, resulting in the dissociation of membrane-bound ligand; and (*b*) preventing the binding and internalization of ligand induced by reexpression of the antigen. We therefore performed internalization studies in the continuous presence of an excess of free MoAb or IT in the medium. We were able to demonstrate the reexpression of CD3 antigen or the expression of newly synthesized CD3 antigen by the observation that the amount of internalized ligands exceeded 2.5 times the maximal number of antigen binding sites under saturating conditions in 24 h. This is in accordance with observations of Krangel[28], who demonstrated a recycling of the CD3/TCR complex to the cell membrane following

endocytosis in the presence of CD3 MoAb. The capacity of the CD3 antigen to induce the internalization of potential cytotoxic ITs for up to 24 h resembles the observation that prolongation of treatment with WT32-ricin A up to 24 h increases cytotoxicity.

Cytotoxicity of WT32-ricin A to T-cells and T-cell lines is enhanced by NH_4Cl[9,22,23] and monensin. Although the mechanism of potentiating is not fully understood, different intracellular compartments and transport mechanisms might be influenced (reviewed in Ref. 29). Previous studies have shown that NH_4Cl and monensin considerably decreased the degradation of 64.1(CD3) by T cells and the T-cell line HPB-ALL.[18,19,24] We demonstrated that NH_4Cl and monensin reduced the degradation of intracellular ^{125}I-WT32 by Jurkat but found differences in their action. Intracellular degradation was decreased during different periods of the continuous incubation, resulting in a temporary accumulation of MoAb. NH_4Cl was most effective during the first 12 h of incubation, whereas monensin decreased the degradation of intracellular ^{125}I-WT32 after 2 h to at least 24 h of incubation. Moreover, NH_4Cl resulted in a higher intracellular accumulation of ligand. As was published before, NH_4Cl did not influence the total amount of internalization.[9,27] Monensin, however, reduced the number of internalized molecules in contrast to observations of Ravel *et al*.[27], who found that monensin had no influence on internalization. Though these results seem to be in favor of NH_4Cl as an enhancing agent, monensin may act more effectively on intracellular handling of ricin A than NH_4Cl, which was confirmed in the cytotoxicity kinetics, since monensin was at least as effective as NH_4Cl.

The efficacy of WT32-ricin A might be extended to cells with low antigen density when treatment is prolonged and either NH_4Cl or monensin is added due to continuous uptake and intracellular accumulation of ITs. Since exposure to ricin A-ITs for 24 h is not harmful to either bone marrow (BM) progenitor cells or stem cells[23], it may now be possible to improve considerably the purging of BM intended for autologous transplantation and therefore the success of this form of therapy.

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CHAPTER 4

EFFECT OF ISOTYPE ON INTERNALIZATION AND CYTOTOXICITY OF CD19-RICIN A-IMMUNOTOXINS

Ypke V.J.M. van Oosterhout

Ingrid E. van den Herik-Oudijk

Hans M.C. Wessels

Theo de Witte

Jan G. J. van de Winkel

Frank W.M.B. Preijers

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ABSTRACT

We analyzed the effect of isotype variation on effectiveness of B-cell CD19 immunotoxins (ITs) by using class switch variants (CLB-B4-IgG1 and CLB-B4-IgG2a) conjugated to ricin A. Notably, IgG1-IT appeared to be ~100-fold more potent than IgG2a-IT toward B-cell lines Daudi and KM3. Binding and internalization studies with ^{125}I-labelled monoclonal antibodies (MoAbs) revealed a higher cellular uptake of IgG1 compared to IgG2a, despite similar binding affinities. Following removal of the Fc-part, both MoAb internalized at the same rate as IgG2a, indicating that the Fc part of IgG1 is involved in enhanced cellular uptake. The involvement of FcγRII (CD32) in this process was demonstrated by a decreased cytotoxicity of IgG1-IT (and not IgG2a-IT) in the presence of FcγRII-blocking MoAbs AT10 or IV.3. To identify the isoform responsible for this phenomenon, internalization of IgG1 and IgG2a in 11 B-cell lines, and malignant B-cells of 8 patients was compared with expression of FcγRII subclasses. In addition to four cell lines (Daudi, KM3, Nalm6, and Raji), the malignant B-cells of two patients showed enhanced uptake of IgG1 relative to IgG2a. Only the FcγRIIa transcript was found in all B-cells. Furthermore, enhanced uptake of IgG1 correlated with rosetting of erythrocytes sensitized with anti-glycophorin A MoAb of IgG1 isotype rather than with FcγRIIa membrane expression levels. These data support the idea that functional FcγRIIa is involved in the enhanced IgG1 uptake observed in a subset of B-cells. Our study, therefore, points to an important role for the Fc region of IT in delivery of cytotoxic effects.

INTRODUCTION

During the past decade, a large number of MoAbs have been conjugated to the highly toxic A chain of the phytolectin ricin in an effort to create tumor-toxic agents. These may be of use for treatment of bone marrow in vitro or may be useful in vivo for patients suffering from hematological malignancies (reviewed in Refs. 1-3). The killing potencies

of such ricin A-chain ITs appeared to vary widely. Firstly, the cytotoxic efficacy of IT depends upon the number of IT molecules internalized by target cells, as well as the intracellular route followed upon internalization.[4-6] Both factors are influenced by the nature of the target cell, type of antigen[7,8], target antigen density[6,9], MoAb binding affinity[9,10], and the IT epitope[11-13]. Furthermore, the biological effects of ITs have been observed to vary depending upon the use of $F(ab')_2$, F(ab'), or intact IgG.[14-16] The effect of variation in isotype on the cytotoxic effectiveness of ITs remained unaddressed.

We constructed two anti-CD19 ITs, which differ in isotype only, by conjugating the murine anti-CD19 MoAb CLB-B4-IgG1 and its isotype-switch variant CLB-B4-IgG2a[17] to ricin A. These ITs differed only in their Fc-parts and expressed identical binding affinities. Interestingly, however, a profound difference in their cytotoxic efficacy was observed using B-cell lines such as Daudi and KM3 as targets. We analyzed the mechanisms underlying the difference in effectiveness between these two IT. Our data point to a role of significant role for the IIa subclass of FcγRII (CD32) molecules expressed on B cells.

MATERIALS AND METHODS

Malignant B-cells and cell lines

Malignant B-cells were obtained from bone marrow or blood of B-cell acute lymphocytic leukemia patients after informed consent. B-cell acute lymphocytic leukemia cells were enriched by Ficoll (1.077 g/ml) density gradient centrifugation. The isolated cell populations consisted of more than 90% of CD19-positive cells as determined by flow cytometric analysis (FCM)(Coulter Epics Elite, Hialeah, FL). The cells were suspended in culture medium and were either used directly or cryopreserved (with 10% DMSO) without loss of functional potential.[18]

The human B-cell lines CRL, Daudi, DOHH2, Fravel, KM3, Nalm6, Raji, Ramos, Ros-1, RPMI, and the T-cell line Jurkat were cultured in a culture medium consising of RPMI 1640 medium (Flow, Irvine, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, Paisley, Scotland), glutamine (2mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified incubator with 5% CO_2 in air at 37°C. 3T6 fibroblasts expressing human FcγRIIa, FcγRIIb1, or FcγRIIb2 molecules were grown in culture medium supplemented with geneticin (0.8 mg/ml; GIBCO BRL, Grand Island, NY).

Monoclonal antibodies

Murine anti-CD19 MoAb CLB-B4-IgG1, and isotype switch-variant CLB-B4-IgG2a[17] were kindly provided by Dr I. Slaper (CLB, Amsterdam, The Netherlands). Hybridoma cells secreting TS2.18 (CD2 and IgG1)[19] and 35.1 (CD2 and IgG2a)[20] were purchased from

American Type Culture Collection (Rockville, MD). The CD2-MoAbs were used as isotype-matched controls. MoAbs were purified from ascites fluid by ammonium sulfate precipitation and affinity chromatography with staphylococcal protein A-sepharose (Pharmacia, Uppsala, Sweden).

MoAb 197 (IgG2a)[21] was used to block FcγRI (CD64), and was obtained from Medarex (Clinton, NJ). IgG and $F(ab')_2$ fragments of MoAb AT10 (IgG1)[22], generously provided by Dr M. Glennie (Tenovus Research Laboratory, SouthHampton, United Kingdom), and IV.3 (IgG2b)[23] from Medarex served to inhibit binding to FcγRII (CD32).

Preparation of $F(ab')_2$ fragments

$F(ab')_2$ fragments of CLB-B4-IgG1 and CLB-B4-IgG2a were prepared using a $F(ab')_2$ preparation kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Briefly, CLB-B4-IgG1 and CLB-B4-IgG2a were incubated with immobilized pepsin at pH 4.2 (20 mM sodium acetate buffer) for 4 and 16 h, respectively. Undigested IgG molecules and Fc-fragments were removed by affinity chromatography with protein A-Sepharose. Remaining fragments smaller than M_r 30,000 were removed by means of a Centriprep 30 concentrator (Amicon, Beverly, MA). The purity of $F(ab')_2$ fragments was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which revealed less then 1% contamination with either intact IgG or Fc fragments (data not shown)

IT preparation

CLB-B4-IgG1, CLB-B4-IgG2a, TS2.18, and 35.1 were conjugated to ricin A (kindly provided by Dr. F. K. Jansen, Centre de Recherches Clin Midy, Montpellier, France) using *N*-succinimydyl 3-(2-pyridyldithio)propionate (SPDP) (Pharmacia), as described.[6] The conjugation ratios of ricin A to MoAb were estimated by measurement of absorbance at 280 nm and radioimmunoasay (RIA) and were determined to be 0.9, 1.1, 0.9, and 0.8 for CLB-B4-IgG1, CLB-B4-IgG2a, TS2.18, and 35.1, respectively. Preservation of antibody-binding activity following conjugation was assessed by FCM.

Cytotoxic potential of IT

Cells in culture medium were distributed into 96-well microtiter plates (Costar, Cambridge, MA) at a concentration of 10^5 cell/well and incubated in triplicate at 37°C with varying concentrations of IT (10^{-8}-10^{-13} M) in the presence or absence of 6 mM NH_4Cl. After 16 h of incubation, 0.5 μCi [^{3}H]leucine (Amersham, Buckinghamshire, United Kingdom) was added, and cells were further incubated at 37°C for another 24 h and harvested. The radioactivity was quantitated, and protein synthesis was expressed as the percentage [^{3}H]leucine incorporation of cells cultured at 37°C without IT.[6]

In some of the cytotoxicity assays, FcγRs were blocked by pre-incubation of target cells with MoAb 197 (purified IgG, 2 μg/ml), AT10 (ascites fluid, diluted 1:500) or IV.3 (purified IgG, 2 μg/ml) for 45 min at 4°C. Cells were washed, and cytotoxicity was assayed

as above. Effectiveness and specificity of blocking with these MoAbs was verified by inhibition of EA-rosetting (Refs. 24 and 25; data not shown).

Radioiodination

MoAbs CLB-B4-IgG1 and CLB-B-4-IgG2a (intact IgG and F(ab')$_2$-fragments), TS2.18 and 35.1 were labelled with Na^{125}I (Amersham) using the Chloramine-T method[26], and purified by chromatography on Sephadex G25 (PD-10; Pharmacia). Eluted fractions containing ^{125}I-IgG and ^{125}I-F(ab')2-fragments, respectively, were pooled, and molecules smaller than M_r 30,000 were removed by Centriprep 30 concentration. Radioactivity due to free ^{125}I and fragments smaller than M_r 30,000 were always less than 0.3% of total radioactivity. The concentration of labelled IgG and F(ab')$_2$ fragments was determined by Mancini assays[27], and specific binding activity was assessed by performing binding studies with limiting amounts of labelled IgG and F(ab')2 fragments.[28]

Scatchard analysis

Suspension of 5 x 10^5 Daudi cells were incubated with various concentrations of ^{125}I-labelled IgG or F(ab')2 fragments for 2 h at 4°C (final volume, 100 µl). Thereafter, cells were washed, and radioactivity was counted to determine the number of cell-associated molecules. Binding to Daudi cells not mediated by antigen was assessed in the presence of a 100-fold excess of unlabelled antibody and was less then 5% of total cell-associated radiolabel for both CD19 MoAbs. Nonspecific binding to incubation tubes was assessed by performing the experiment in the absence of targets. The affinity constant (*K*a) and the number of bound molecules/cell under saturating conditions were calculated according to Scatchard.[29]

Internalization of ^{125}I-IgG and ^{125}I-F(ab')2

The cellular uptake and processing of ^{125}I-labelled IgG, or F(ab')2 fragments, were determined as described.[28] Briefly, a suspension of 5 x 10^5 cells was incubated with 10^{-8} M ^{125}I-IgG or ^{125}I-F(ab')$_2$ in culture medium at 37°C (final volume, 100 µl). Following different times, cells were pelleted, and the amount of (internalized, and subsequently) degraded and exocytosed MoAb was estimated by measurement of TCA (25%)-soluble radioactivity of the supernatant. Cells were then washed twice and assessed for total cell-associated (surface-bound, and intracellular) radioactivity. Surface radioactivity was subsequently released by incubation with isotonic glycine-HCl buffer (pH2; 4°C for 30 min); then cells were washed, and intracellular radioactivity was determined. Nonantigen-mediated binding and internalization of ^{125}I-ligand was assessed in the presence of a 100-fold excess of unlabelled antibody and was typically less then 5% of total cell-associated radiolabel. Experiments perfomed at 4°C were used to correct for surface radioactivity not removable by glycine-HCl buffer (usually <5%) and background radioactivity of the TCA-soluble fraction. Extracellular degradation at 37°C could be excluded since negligible radioactivity was found in the TCA-soluble fraction of T-cell line Jurkat, which does not express CD19, and in purified surface membranes from dis-

rupted Daudi or KM3 cells following incubation with ^{125}I-IgG or ^{125}I-F(ab')$_2$.

Internalization was defined as the total number of molecules transported across the cell membrane, and calculated by summation of TCA-soluble radioactivity to intracellular radioactivity.

Detection of FcγRIIa, FcγRIIb1 and FcγRIIb2 transcripts in the panel of B-cell lines

The presence of FcγRIIa, FcγRIIb1 and FcγRIIb2 transcripts in the panel of B-cell lines was assessed by RT-PCR as described before.[30] Briefly, total cellular RNA was extracted from 5 x 10^6 cells using the RNazol B isolation method[31], concentrated by ethanol precipitation, resuspended in diethylpyrocarbonated water, and stored at -70°C. Total cellular RNA (5 μg) was reverse transcribed and the presence of FcγRIIa and FcγRIIb cDNA were amplified by PCR, using FcγRIIa- and FcγRIIb-specific primer pairs. PCR products were analyzed by electrophoresis on 1.8% (w/v) agarose gels stained with ethidium bromide, followed by Southern blotting to Zeta-probe nylon membranes (Bio-rad). To detect FcγRIIa and FcγRIIb PCR products, blots were hybridized overnight with FcγRIIa- and FcγRIIb-specific ^{32}P-labelled oligonucleotide probes, which were then washed, and analyzed using a 400A Phosphor-imager System (Molecular Dynamics, Sunnyvale, CA). cDNA of FcγRIIa, FcγRIIb1, and FcγRIIb2 transfectants served as controls.

Membrane expression of FcγRII

Surface expression levels of FcγRII (CD32) were determined via indirect immunofluorescence measured by FCM. Cells in phosphate-buffered saline (PBS) supplemented with 0.1% (w/v) bovine serum albumine and 0.1% sodium azide were incubated with MoAb IV.3 (2 μg/ml) for 45 min at 4°C and then washed and incubated with fluorescein isothiocyanate-labeled F(ab')$_2$ fragments of goat-anti-mouse IgG (Nutacon, American Qualex Division, Amsterdam, the Netherlands) for another 45 min at 4°C. Subsequently, cells were washed and the mean fluorescence intensity (MFI) of 10,000 cells was evaluated by FCM and expressed in arbitrary units.

EA-IgG rosetting

Rosette formation between B-cells and E sensitized with anti-glycophorin A switch-variant MoAb of IgG1-isotype[32] was performed as described before.[30] Briefly, a 0.5% suspension of human E in PBS was incubated for 30 min at 37°C with the anti-glycophorin A MoAb and then washed and suspended in RPMI 1640-5% FCS. Fifty μl were then added to 50 μl B-cells (4 x 10^5), centrifugated (4 min at 40 x g), and incubated for 1 h at 4°C. Cells with at least three bound E were microscopically scored as EA-rosettes.

Statistical analysis

Statistical evaluation of results was performed using the Spearman correlation.

RESULTS

Cytotoxicity of CLB-B4-ricin A isotype-switch variants

The efficacy of the class switch variant ITs was determined by protein synthesis inhibition assayed with B-cell lines Daudi and KM3, which show high and intermediate membrane-expression levels of CD19, respectively. Both ITs demonstrated poor cytotoxicity (ID_{50}s of $\geq 10^{-8}$ M) in the absence of NH_4Cl. In the presence of NH_4Cl (6 mM) which enhances IT cytotoxicity[33], the IgG1-IT became about 100 times more potent than IgG2a-IT (ID_{50}s of ~10^{-10} M, and 10^{-8} M, respectively). Neither IT, even in the presence of NH_4Cl, showed any cytotoxicity against T-cell line Jurkat, which lacks expression of CD19 (Figure 1).

In order to confirm that the cytotoxic effect of the CLB-B4-IT is mediated through the CD19 antigen, the blocking effect of free CD19 MoAb was examined. The addition of a 100-fold excess of CLB-B4-IgG2a completely blocked the cytotoxic action of the CLB-B4-IT against Daudi cells ($ID_{50} > 10^{-8}$ M in the presence of NH_4Cl for both the IgG1-IT and IgG2a-IT; data not shown). Moreover, non-binding control IT TS2.18-ricin A (CD2 and IgG1) and 35.1-ricin A (CD2 and IgG2a) demonstrated no cytotoxicity against Daudi cells (data not shown).

Scatchard analysis of the CLB-B4 IgGs and F(ab')$_2$ fragments

In order to determine whether the observed differences in cytotoxicity were attributable to differences in affinity, we performed binding studies with both MoAb (and their F(ab')$_2$-fragments) on Daudi cells. Both affinity (~8 x 10^9 M^{-1}), and number of cell-bound molecules (~67,000) were found comparable for both MoAbs (and their F(ab')$_2$ fragments; Table 1).

Table 1 *Scatchard analysis of CLB-B4-IgG1 and CLB-B4-IgG2a binding to Daudi cells[a]*

	CLB-B4-IgG1		CLB-B4-IgG2a	
Antibody form	K_a (x 10^{-9} M)	Molecules Bound/cell (x 10^{-4})	K_a (x 10^{-9} M)	Molecules Bound/cell (x 10^{-4})
IgG	7.1 ± 1.0	6.7 ± 1.2	8.3 ± 2.1	6.4 ± 0.8
F(ab')$_2$	8.6 ± 1.4	6.9 ± 0.9	7.5 ± 1.8	6.9 ± 1.2

[a]Cells were incubated with various concentrations of ^{125}I-MoAb for 2 h at 4°C and then washed and assessed for membrane-bound radioactivity to calculate the affinity constant (K_a); the number of bound molecules/cell were counted under saturating conditions according to Scatchard (29). The mean ± SD of three independent experiments is shown.

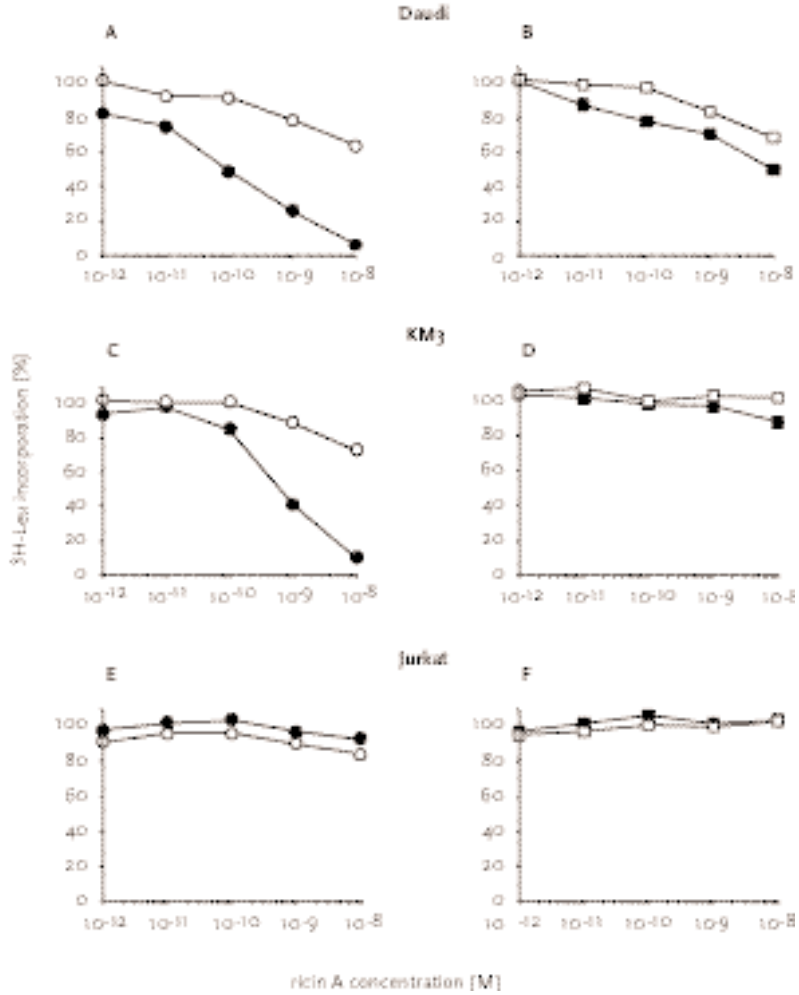

Figure 1 *Cytotoxicity mediated by CLB-B4-ricin A-IgG1 and CLB-B4-ricin A-IgG2a*

Daudi (A and B), KM3 (C and D), or Jurkat (E and F) cells were incubated with various concentrations of CLB-B4-ricin A-IgG1 (A, C, and E) or CLB-B4-ricin A-IgG2a (B, D, and F) in the absence (○,□) or presence (●,■) of 6 mM NH_4Cl at 37°C. After 16 h, cells were washed and cultured with [^{3}H]leucine for another 24 h. Protein synthesis is expressed as the percentage [^{3}H]leucine incorporation relative to cells cultured in medium alone (control cells). Control Daudi and KM3 cells incorporated ~15,000 cpm, and Jurkat cells incorporated ~20,000 cpm. Background incorporation approximated 100 cpm. Assays were performed in triplicate (n = 3), and SD was <10%.

Binding and internalization kinetics of CLB-B4-IgG1 and CLB-B4-IgG2a

Because the efficacy of IT strongly depends on numbers of internalized molecules[6], we studied binding and internalization kinetics of the switch variants. Following incubation

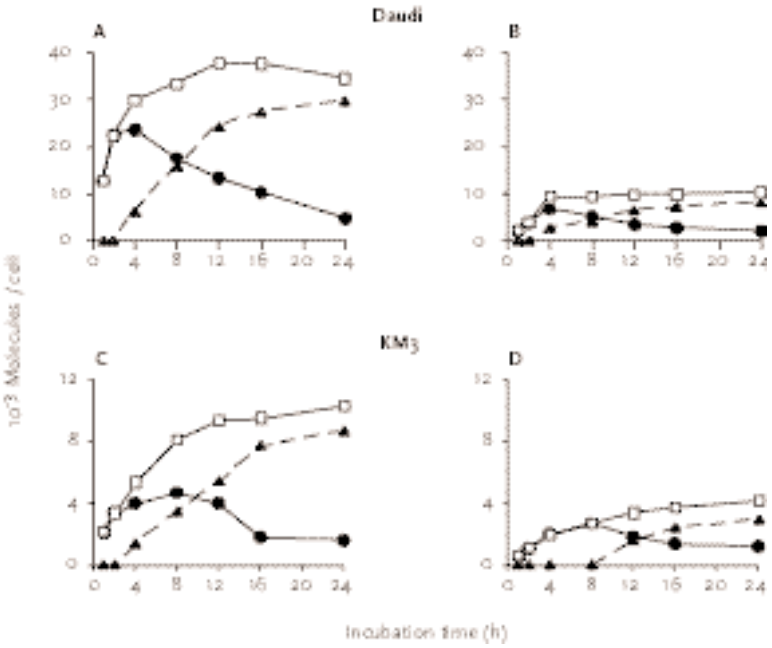

Figure 2 *Cellular uptake and processing of CLB-B4-IgG1 and CLB-B4-IgG2a*

Daudi (A and B) and KM3 (C and D) were incubated with ^{125}I-labeled CLB-B4-IgG1 (A and C) or CLB-B4-IgG2a (B and D) for various times at 37°C. Intracellularly degraded and subsequently exocytosed molecules (TCA-soluble radioactivity of supernatants; ▲) and intracellular molecules (cell associated radioactivity not-removable by glycine buffer; ●) were assessed as described in "Material and Methods". Internalized molecules were determined as the sum of intracellular and intracellularly degraded and subsequently exocytosed molecules (□). Assays were performed in triplicate (n = 3), and SD was <10%. One of three concordant experiments is shown.

at 4°C with 10^{-8} M, equal amounts of IgG1 and IgG2a MoAbs were bound to the cell membranes (Daudi and KM3 cells, ~40,000 and ~15,000 molecules, respectively; data not shown). Incubation at 37°C, however, resulted in consistent internalization differences (Figure 2). Daudi cells internalized 3.5 times and KM3 cells 2.5 times more IgG1 MoAb than IgG2a MoAb (Daudi, 35,000 IgG1 molecules and 10,000 IgG2a molecules; KM3, 10,000 IgG1 molecules and 4,000 IgG2a molecules) during a 12-h incubation. The highest number of intracellularly present molecules was reached after 4 and 8 h for Daudi (~25,000 IgG1 molecules and 7,500 IgG2a-molecules), and KM3 cells (~4,000 IgG1 molecules and 3000 IgG2a molecules), respectively. Thereafter the number of intracellularly present molecules declined as a result of exocytosis (of intracellularly degraded MoAb).

Because the switch-variants differ only in subclass, we next tested the effect of removing the CD19-Fc fragment on internalization. Daudi cells were incubated with 10^{-8} M of

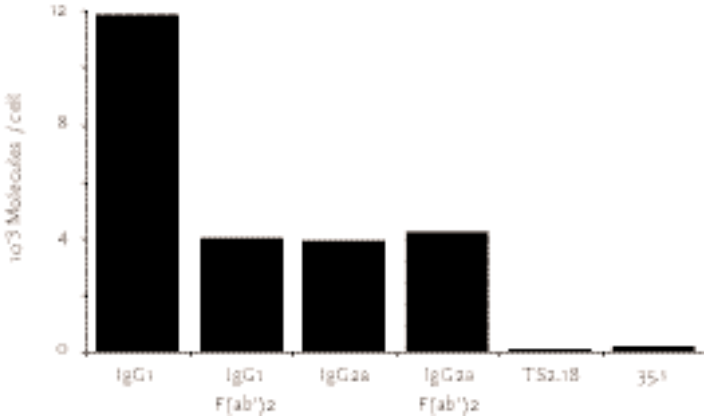

Figure 3 *Effect of isotype on internalization*

Daudi cells were incubated for 1 h at 37°C/4°C with ^{125}I-labeled IgG or F(ab')$_2$-fragments of CLB-B4-IgG1 and CLB-B4-IgG2a, or with isotype-matched MoAb TS2.18 (CD2 and IgG1) or 35.1 (CD2 and IgG2a). Data are presented as molecules/cell incubated at 37°C (corrected for cells treated at 4°C) as described in "Material and Methods". Experiments were performed in triplicate, and SD was <10%. One of three experiments yielding similar data is shown.

intact IgG or F(ab')$_2$ fragments of CLB-B4 for 1 h at 37°C; then the amount internalized molecules was determined (Figure 3). Both the IgG1- and the IgG2a-F(ab')$_2$-fragments were internalized to a comparable extent as intact IgG2a MoAb, indicating that the Fc part of the IgG1 MoAb was responsible for the observed increase in internalization. The binding of CD19 antigen appeared to be a prerequisite because no cellular uptake could

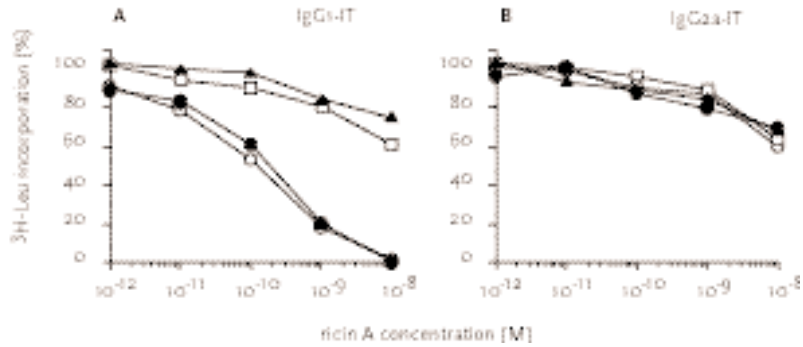

Figure 4 *Effect of FcγR-blocking MoAb on IT-cytotoxicity*

Daudi cells were incubated with various concentrations of the IgG1-IT (A) or IgG2a-IT (B) with NH_4Cl, in the absence (○) or presence (●) of FcγRI-blocking MoAb 197, or FcγRII-blocking MoAb AT10 (▲), or IV.3 (□) at 37°C. After 16 h, cells were washed and cultured with [^{3}H]leucine for another 24 h. Protein synthesis is expressed as the percentage [^{3}H]leucine incorporation relative to cells cultured in medium lacking IT. Untreated Daudi cells incorporated ~15,000 cpm. Background incorporation was 100 cpm. Assays were performed in triplicate, and SD was <10%. The experiment was repeated three times, yielding almost identical results.

be observed of isotype-matched irrelevant MoAb TS2.18 (CD2 and IgG1) or of 35.1 (CD2 and IgG2a).

Involvement of FcγRII in CLB-B4-ricin A cytotoxicity

Because the involvement of the IgG1-Fc fragment suggested an interaction with receptors for the Fc domain of IgG, we performed cytotoxicity assays with Daudi cells in the presence of FcγR-blocking MoAb. Addition of FcγRI-blocking MoAb 197 did not affect cytotoxicity of the IgG1-IT or IgG2a-IT. Coincubation of cells with FcγRII-blocking MoAb AT10 or IV.3 reduced the effectiveness of IgG1-IT without affecting IgG2a-IT cytotoxicity (Figure 4). Addition of AT10 $F(ab')_2$ fragments (2 μg/ml) resulted in a similar blocking to AT10 IgG (data not shown; $n = 3$), excluding the reduced cytotoxicity to be caused by the Fc part of this FcγRII-blocking MoAb. These results indicated that FcγRII is involved in the observed differences between the class switch ITs.

Table 2 *Relation between CLB-B4 internalization, FcγRIIa expression levels, and EA-rosette formation by B-cell lines and patient cells*

	Internalization[a] (Molecules/cell)				
Cells	IgG1	IgG2a	IgG1/IgG2a[d]	FcγRIIa expression[b] (MFI)	EA-rossetting[c] (%)
CRL	2,879	2,624	1.1	14.9	0
Daudi	9,540	3,085	3.1	18.8	96
DOHH2	11,245	11,063	1.0	1.1	0
Fravel	2,328	2,154	1.1	18.7	0
KM3	2,008	800	2.5	1.9	0
Nalm6	10,381	2,002	3.5	10.4	95
Raji	8,550	5,700	1.5	11.8	10
Ramos	11,251	11,941	1.0	0.4	0
REH	1,009	900	1.1	1.6	0
Ros1	4,532	4,498	1.0	22.0	0
RPMI1788	1,259	1,290	1.0	5.1	0
Patient 1	1,019	917	1.1	6.3	0
Patient 2	3,192	3,002	1.1	2.1	2
Patient 3	2,201	1,928	1.1	1.0	0
Patient 4	2,675	1,574	1.7	17.8	20
Patient 5	3,172	1,233	2.6	3.5	60
Patient 6	4,841	4,586	1.1	15.0	0
Patient 7	1,775	1,714	1.0	1.0	0
Patient 8	515	498	1.0	8.5	0

[a]*Cells were incubated for 1 h with 10^{-8} M ^{125}I-labeled MoAb at 37°C; then the number of internalized molecules/cell was determined as described in "Materials and Methods". The mean of three independent experiments is shown; SD < 10%.* [b]*FcγRII-expression levels were determined by indirect immunofluorescence. Cells were incubated with MoAb IV.3 (predominantly interacting with FcγRIIa) for 45 min at 4°C, washed and cultured for another 45 min with fluorescein isothiocyanate-labeled $F(ab')_2$ fraction of anti-mouse immunoglobulin antiserum. Data shown represent the MFI of 10,000 cells.* [c]*EA-rosette formation was used to estimate functional binding capacity of FcγRIIa. Cells were incubated with EA sensitized with IgG1 for 1 h at 4°C and washed and assessed for EA-rosettes. Data represent the percentage of cells with three or more bound EA-IgG. The mean of three experiments is shown; SD < 10%.* [d]*Ratio of internalized IgG1 and IgG2a molecules upon incubation for 1h at 37°C.*

Relation between internalization, FcγRII-isoform expression and EA-IgG rosette formation

To determine the FcγRII-isoform involved in the internalization of the IgG1 MoAb, we studied the relation between enhancement of IgG1 uptake and expression of the different isoforms. The enhancement of IgG1 uptake in 11 B-cell lines and malignant B-cells derived from 8 patients (determined after 1 h at 37°C) was expressed as the ratio of internalized IgG1 and IgG2a molecules (Table 2). As in some cases, the number of internalized molecules was rather low; nonspecific endocytosis due to a fluid phase uptake was examined with MoAbs TS2.18 (CD2 and IgG1) and 35.1 (CD2 and IgG2a). No cellular uptake of these control antibodies could be observed (data not shown). Besides Daudi, KM3, Nalm6 and Raji cells, malignant B-cells from two of the patients (patients nos. 4 and 5) showed an enhanced internalization of CD19-IgG1 molecules.

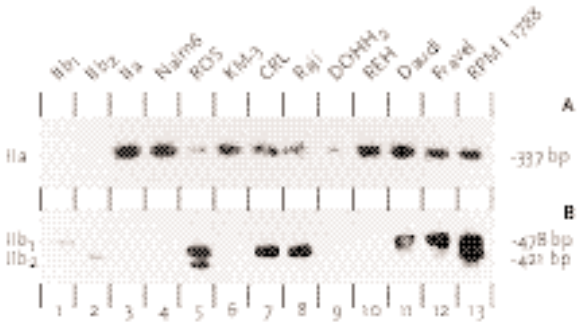

Figure 5 *Expression of FcγRIIa, FcγRIIb1 and FcγRIIb2 transcripts in a panel of B-cell lines assessed by reverse transcription-PCR*

RNA was isolated and reverse transcribed, and FcγRIIa and FcγRIIb transcripts were selectively amplified (described in "Material and Methods"). Products were run on a 1.8% agarose gel, blotted, and hybridized with isoform-specific oligonucleotide probes. FcγRIIa, FcγRIIb1, and FcγRIIb2 transfectants served as controls (Lanes 1-3). Blots were probed with either ^{32}P-labelled FcγRIIa (A) or FcγRIIb (B) specific oligonucleotides.

The expression of various FcγRII transcripts in the panel of B-cell lines was analyzed by performing an FcγRIIa- and FcγRIIb-specific PCR. Total cellular RNA was isolated and reverse transcribed to cDNA; specific primer pairs were used to amplify FcγRIIa, FcγRIIb1 and FcγRIIb2 transcripts (Figure 5). FcγRIIa was found to be present in all B-cell lines tested. The FcγRIIb1 and FcγRIIb2 expression levels varied between the different B-cell lines. Notably, FcγRIIa represented the sole FcγRII transcript present in Nalm6, KM3, DOHH2 and REH cells, suggesting that: (*a*) the FcγRIIa isoform is involved in enhancement of IgG1 uptake (Nalm6 and KM3 showing IgG1:IgG2a ratios of 3.5 and 2.5, respectively); and (*b*) FcγRIIa RNA expression as such is not indicative for enhanced IgG1 uptake (DOHH2 and REH, IgG1:IgG2a ratios of ~1).

Because the presence of RNA transcripts does not necessarily imply membrane expression of the receptor, we determined the membrane-expression levels of FcgRIIa using MoAb IV.3, which predominantly interacts with FcγRIIa (34; Table 2). Cell lines DOHH2, KM3, Ramos, REH, and B cells derived from patients 1,2,3,5, and 7 showed no or poor expression. All other cells showed intermediate to high expression. The relation between membrane expression levels and enhanced uptake of CD19-IgG1 is plotted in Figure 6. No correlation could be found ($r = 0.32$; $P < 0.185$; Fig. 6A).

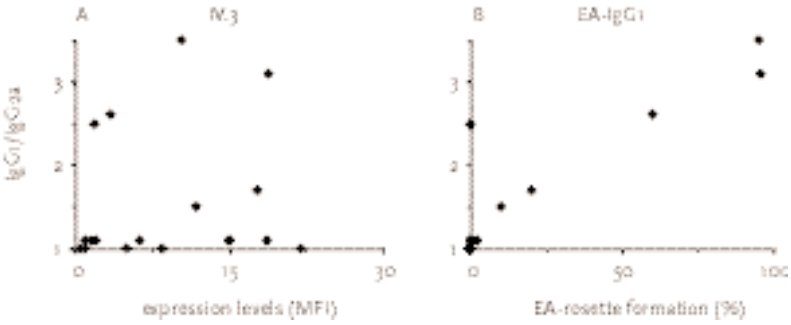

Figure 6 *Relation between enhancement of IgG1 uptake, FcγRII expression levels, and EA-rosette formation*

To assay FcγRII expression levels (A), cells (as indicated in Table 2) were incubated with MoAb IV.3 for 45 min at 4°C, washed and cultured for another 45 min with fluorescein isothiocyanate-labeled anti-mouse serum. Data represent the mean fluorescence intensities (MFI). EA-rosette formation (B) was determined after incubation of cells with EA-IgG1 for 1 h at 37°C. Data represent the percentage of cells (indicated in Table 2) with three or more bound EA-IgG indicator cells. Assays were performed in duplicate. The experiment was repeated three times, yielding almost identical results.

The relation between expression levels and enhanced CD19-IgG1 uptake may be complicated by membrane-expression of "nonfunctional" FcγRIIa molecules. Therefore, we assessed functional IgG binding capacity of the FcγRIIa molecules by determining EA-rosette formation using IgG1-sensitized E (EA-IgG1; Table 2). A highly significant correlation between enhanced IgG1 uptake and EA-rosette formation was found ($r = 0.76$; $P < 0.001$; Fig. 6B). Remarkably, KM3 cells showed an enhanced uptake of IgG1 (ratio 2.5) without forming rosettes. This may have been due to the exceptionally low FcγRII-expression level on KM3 (MFI, 2.2). These results suggest that FcγRIIa molecules capable of IgG1 are involved in the enhancement of internalization observed upon interaction of cells with CD19-IgG1-IT.

DISCUSSION

Several reports addressed the use of CD19 MoAb-toxin conjugates as potent and specific reagents for neoplastic B-cell elimination. These include MoAb HD37 conjugated to ricin A[10], to saporin[35], as well as MoAb B4 conjugated to blocked ricin[36] and MoAb B43 conjugated to pokeweed antiviral protein (PAP)[37], all of the mouse IgG1 isotype. In the present study, we evaluated the cytotoxic potency of CD19 class switch variants CLB-B4-IgG1 and CLB-B4-IgG2a upon conjugation to ricin A. The IgG1-IT required the presence of the cytotoxicity enhancer NH_4Cl to show similar cytotoxicity as reported for HD37-ricin A against Daudi cells (ID50s of ~10^{-10} M; Fig. 1)[38]. This difference in potency is probably due to a variation in epitope specificifty and/or binding affinity. More surprisingly, in view of both MoAbs sharing identical epitopes and binding characteristics, the CLB-B4-IgG2a-IT appeared to be ~100-fold less cytotoxic than the CLB-B4-IgG1-IT when assayed on Daudi and KM3 cells (ID50 of ~10^{-8} M).

Internalization studies with ^{125}I-labelled MoAb and $F(ab')_2$ fragments revealed that the higher efficacy of the IgG1-IT is attributable to the IgG1 Fc fragment, enhancing the cellular uptake following membrane binding (Figs. 2 and 3). The enhancement of IgG1 uptake was observed in 4 of 11 B-cell lines and in B-cells of 2 of 8 patients examined, suggesting the involvement of a heterogeneously expressed receptor on B-cells. A likely candidate to be involved in the enhanced uptake of IgG1 may be FcγRII (CD32), which is both present on B-cells and capable of interacting with IgG1.[30,39] By interaction with the Fc part of membrane-bound CD19-IgG1, FcγRII may be capable of supporting internalization of antigen-MoAb complexes, analogous to the mechanisms described by Kurlander.[40] This hypothesis was supported by the observation that incubation with FcγRII-blocking MoAb strongly reduced the cytotoxicity of the IgG1-IT (and not IgG2a-IT; Fig. 4). The increase in potency resulting in the interaction of the IgG1-Fc part with FcγRII (~100-fold; Fig. 1) is tremendous, considering the relatively modest increase in internalization (~2.5-3.5-fold; Fig. 2). Further studies are needed to address this disproportional increase in cytotoxicity, but it is tempting to speculate that the interaction with FcγRII alters the intracellular routing and/or handling of the antigen-IT complex in a way more favorable for ricin A to exert its cytotoxic action.

Expression of the FcγRII-isoform involved in the internalization of IgG1-IT in B cells was assayed by isoform-specific PCR experiments. Notably, FcγRIIa was the only isoform expressed by cell lines KM3 and Nalm6, showing enhanced IgG1-uptake. The strong correlation of enhancement of IgG1-uptake with EA-IgG1 rosetting, rather than with expression levels determined using MoAb IV.3 (predominantly interacting with FcγRIIa)[34], suggested that part of the expressed FcγRIIa molecules are incapable of IgG1-binding (and, thereby, incompetent to promote cellular uptake). This is in accordance with observations of Koenderman *et al*, who demonstrated that not all FcγRIIa molecules expressed by eosinophils are able to bind EA-IgG.[25] Remarkably, the cell line KM3 did

not form rosettes with EA-IgG1 but does show enhanced IgG1-uptake. In our view, this may be attributable to the exceptionally low FcγRIIa expression levels on KM3, which might be sufficient for enhancing IgG1 uptake in a catalytic manner (Table 2), but too low to support stable EA-IgG1 binding in EA-rosette experiments (Fig. 6B).

Several factors have been described to affect internalization and intracellular handling and, thereby, the cytotoxicity of ricin A ITs. Among these are the nature of the target cell and antigen[7,8], antigen density[6,9], binding affinity of the MoAb[9,10], and the epitope to which the IT is directed[11-13]. Our observation of class switch variant ITs displaying different levels of cytotoxicities suggests also that the MoAb isotype is important for the efficacy of anti-B-cell ITs. Additional studies, however, are needed to address whether or not this phenomenon is restricted to CD19-ITs.

From our study, we conclude that the Fc part of ITs may be a parameter of importance for the efficacy of anti-B-cell ITs. Selection of the most effective isotype may improve the killing potency of an IT and, therefore, the success of these reagents in human therapy.

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CHAPTER 5

FLOW CYTOMETRIC EVALUATION OF IMMUNOTOXIN INDUCED CELL KILL

Ypke V.J.M. van Oosterhout

Peter J. van Horssen

Arie H.M. Pennings

J. Liesbeth van Emst

Theo de Witte

Frank W.M.B. Preijers

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ABSTRACT

Immuntoxins (ITs) represent an attractive drug targeting concept with therapeutic potential for the treatment of a variety of malignancies and immunological disorders. Prior to their clinical evaluation, the reactivity of IT towards target and non-target cells has to be extensively evaluated. In this report, a method is described for the preclinical evaluation of IT-induced target cell killing. This method is based on the flow cytometric (FCM) identification of viable cells escaping IT treatment. The simultaneous use of two viability markers, propidium iodine (PI) and calceine (Calc), minimizes the occurrence of falsely postive/negative cells due to autofluorescence, or extensive DNA-disintegration preventing PI-incorporation. The admixture of a fixed concentration of inert beads prior to analysis enables the precise quantification of surviving cells. Using this method, it appeared that IT-induced cell kill is not immediately accompanied by the physical disruption of the cell membrane. An IT-free lag period of four days proves essential for full exposure of efficacy. With a 1:9 mixture of leukemic Ramos cells and normal bone marrow cells, it was demonstrated that the method can also be used for the evaluation of IT-based killing of non-clonogenic target cells in a heterogeneous cell sample. With its capacity of monitoring target cell kill in freshly derived heterogeneous patient samples, the flow cytometric assay forms a valuable addition to the conventional clonogenic assays en protein synthesis inhibition assays.

INTRODUCTION

Immunotoxins (ITs) are hybrid molecules consisting of a cell-specific compound, mostly monoclonal antibodies (MoAbs), conjugated to a potent bacterial or plant derived toxin, *e.g.* modified diphteria toxin or the A chain of plant toxin ricin. Several studies demonstrated the potential of ITs to selectively kill target cells in a highly efficient manner.[1-3] Moreover, the past two decades, ITs have been tested both in animals and

human studies for the treatment of life-threatening diseases, including various cancers and immunological disorders.[4-11]

Prior to its clinical use, ITs have to be extensively screened for reactivity against target and non-target cells. Their potency is regularly expressed as the dose resulting in 50% inhibition of protein synthesis (ID_{50}). In general, ITs having an ID_{50} of 10^{-10} M or less are considered suitable for clinical use, supposed they do not cross-react with life-sustaining tissues.[12] It should be realized, though, that the presence of a small fraction of IT-resistant target cells might give rise to recurrence of the disease without having much impact on the ID_{50}. Therefore, in designing the optimal therapy, it is also important to determine the number of cells escaping various treatment regimens. So far, clonogenic assays have proven to be an useful tool for an accurate quantification of cells resistant to IT-treatment, especially when using cell lines as target.[13-15] Unfortunately, the in vitro clonogenicity of patient derived target-cells is often too low to perform a reliable clonogenic assay. Because of this limitation, a quantitative flowcytometric (FCM) method was developed capable of determining IT-induced cell kill in freshly derived patient-samples as well. This study determines the prerequisites for a reliable quantification of surviving target-cells in a heterogeneous population such as human bone marrow.

MATERIALS AND METHODS

Malignant B-cell lines and bone marrow cells

Human B-cell lines Daudi, KM3 and Ramos were obtained from the American Type Culture Collection (ATTC, Rockville, MD). Normal bone marrow cells were obtained from patients undergoing cardiac surgery, after informed consent. Erythrocytes and mature granulocytes were removed by Ficoll (1.077 g/ml) density gradient centrifugation. Mononuclear cells were suspended in culture medium and were either used directly, or cryopreserved (10% dimethyl sulfoxide) without loss of functional potential.[16]

Cells were cultured in medium consisting of RPMI 1640 (Flow, Irvine, Scotland) supplemented with 10% heat-inactivated FCS (Gibco, Paisley, Scotland), glutamine (2mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified incubator with 5% CO_2 in air at 37°C.

Immunotoxins (IT)

MoAb CLB-B4 (anti-CD19, IgG1) and CLB-B-ly/1 (anti-CD22, IgG1) were obtained from the Central Laboratory of the Red Cross Blood Bank (Amsterdam, the Netherlands). Recombinant ricin A was generously provided by Zeneca Pharmaceuticals (Macclesfield, UK). ITs were constructed by biochemical linkage of MoAb and ricin A using *N*-succinimydyl-3-(2-pyridyldithio)propionate (SPDP) (Pierce, Rockford, IL), as previously described.[13] Following conjugation, preservation of MoAb-binding activity was assessed

by FCM (Coulter Epics Elite, Hialeah, FL). Retention of ricin A activity was determined in a rabbit reticulocyte lysate assay, as described by Press *et al*.[17]

IT-treatment

Cells in culture medium at a concentration of 10^6 cells/ml were incubated with IT in triplicate at 37°C. IT concentrations were expressed in molar, ranging from 10^{-13}-10^{-8} M. In some experiments, cytotoxicity enhancers NH_4Cl (6 mM) or nigericin (10 nM) were added to the culture medium to enhance the cytotoxity of the ricin A-IT. After 16 h of IT-treatment, cells were washed and assayed for the number of surviving cells by either flow cytometry or a clonogenic assays.

Flow cytometric evaluation of surviving cells

Following IT-treatment, cells were cultured for varying days in IT-free culture medium at 37°C. After this lag period, cells were incubated with 2 μg/ml propidium iodine (PI) (Molecular Probes, Junction City, OR) for 30 minutes at room temperature to stain death cells. In later experiments, also 2 μg/ml calcein AM (Calc) (Molecular Probes) was added as an additional viability marker. Samples were then analyzed in triplicate on a flow cytometer (Coulter Epics Elite, Hialeah, FL) using a minimum of 10,000 cells. Viable cells were identified as being PI-negative and, if applicable, Calc-positive. Overlap of emission spectra of PI and Calc was adjusted by electronic compensation using single-labeled samples. Prior to FCM analysis, a fixed amount of inert beads (DNA-check or Flow-Count fluorospheres, Coulter) was added to each sample (*e.g.* $5x10^4$ beads/ml) to enable the quantification of surviving cells. To prevent sedimentation of the beads, the FCM analysis was performed under continuous gently shaking of the sample.

Clonogenic assays

The clonogenic capacity of IT-treated cells was determined as described earlier.[13,14] Briefly, IT-treated cells were resuspended in cloning medium consisting of RPMI 1640 supplemented with 1 mM α-ketoglutarate (Sigma, St. Louis, MO), 1 mM sodium oxaloacetate (Sigma), 5% fetal calf serum, and 10% heat inactivated horse serum (Hyclone, Logan, UT). Varying numbers of cells (10^2-10^6) were seeded in double layer agarose (3%) and cultured for 15 days at 37°C. Absolute numbers of surviving cells were calculated from the number of colonies, the number of plated cells and the cloning frequency of untreated cells (12% for Daudi and 28% for KM3).

Detection of IT-efficacy in a heterogeneous sample

Ramos cells were stained with the green-fluorescent membrane intercalating dye D-275 (Molecular probes, Eugene, OR), according to the manufacturer's protocol. Labeled Ramos cells were mixed in a 1:9 ratio with normal bone marrow and subsequently treated with IT and analyzed by FCM as described above. Overlap of emission spectra of PI and D-275 could be adjusted by electronic compensation using single-labeled samples.

RESULTS

Kinetics of IT-Induced Cell Kill as Determined by FCM

PI incorporates in the DNA of dead cells after passing through their compromised membranes. For a reliable assessment of IT-induced cell kill it is essential to determine the 'lag-period' between the irreversible inhibition of protein synthesis and the resulting physical disruption of the cell membranes.

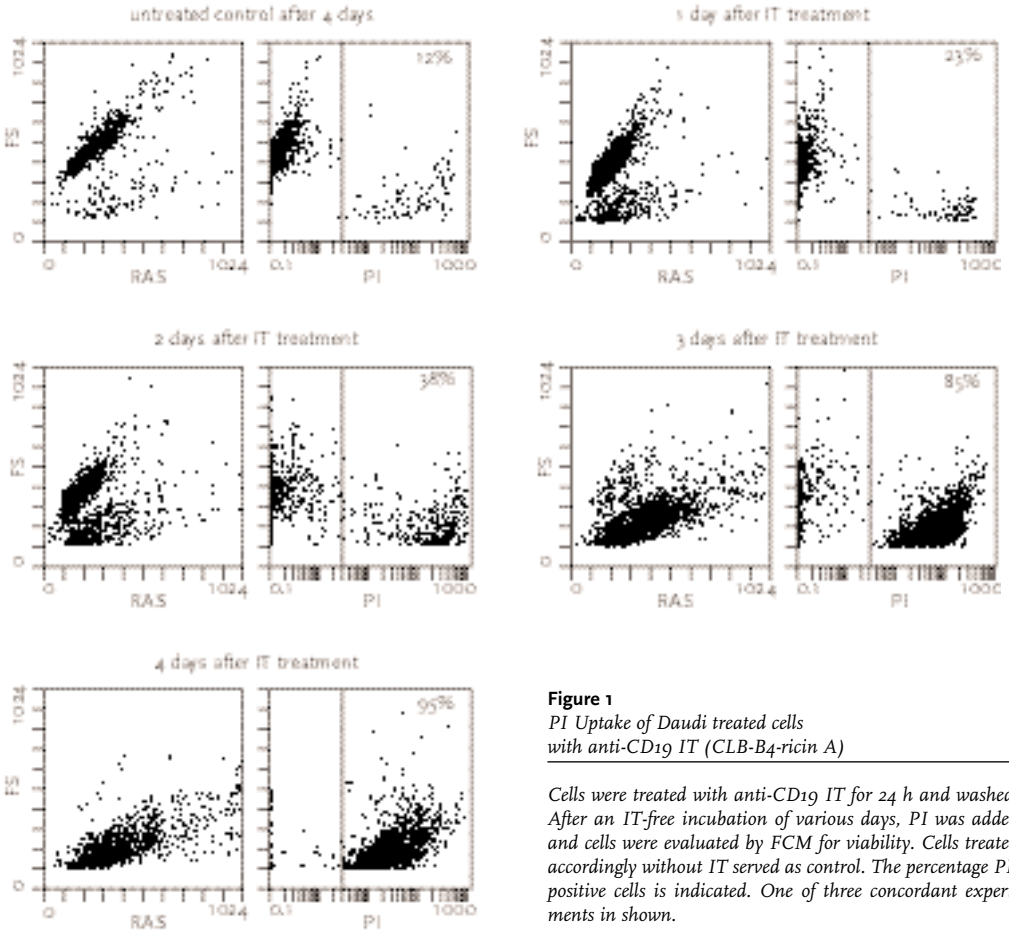

Figure 1
PI Uptake of Daudi treated cells with anti-CD19 IT (CLB-B4-ricin A)

Cells were treated with anti-CD19 IT for 24 h and washed. After an IT-free incubation of various days, PI was added and cells were evaluated by FCM for viability. Cells treated accordingly without IT served as control. The percentage PI-positive cells is indicated. One of three concordant experiments in shown.

Figure 1 illustrates the fate of Daudi cells treated with CLB-B4-ricine A (anti-CD19) in the presence of NH_4Cl as enhancer of cytotoxicity. Prolongation of the post-treatment (IT-free) incubation period up to four days resulted in a progressive increase in the number of dead or dying cells, characterized by a strongly reduced forward scatter. This

change in light-scatter signals was accompanied by a disturbance of the physical integrity of the cell membranes, as reflected by the resulting PI^+ phenotype. The maximal number of dead cells was observed four days after treatment with CLB-B4-ricin A (95% PI^+), and did not increase upon further prolongation of the post-treatment period. The control sample treated with unconjugated MoAb CLB-B4 and ricin A, contained about 10% dead cells following the same incubation period. Similar patterns were observed with different combinations of ITs and cell lines (data not shown, n = 5).

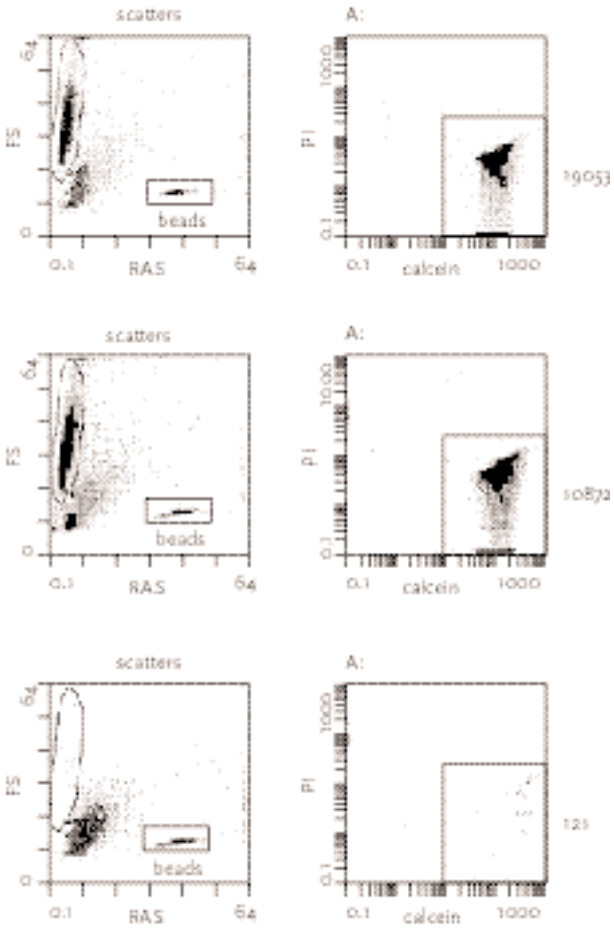

Figure 2
Quantification of IT-induced cell kill

Ramos cells were incubated with a suboptimal concentration anti-CD22 IT (CLB-B-Ly/1-ricin A, $5x10^{-11}$ M), in the presence (C) or absence (B) of nigericin (10 nM). Cells incubated with culture medium served as control (A). After 24 h, cells were washed and incubated without IT for an additional 4 days. Subsequently, cells were stained with PI and Calc and analyzed by FCM for viability ($PI^-/Calc^+$) in the presence of a fixed concentration of inert beads ($5x10^4$/ml). Analysis was stopped when 1,000 beads had passed the flow cell. One of three concordant experiments is shown.

Quantification of surviving cells

Treatment with IT may result in the elimination of several logs of antigen-bearing target cells, especially in the presence of cytotoxicity enhancers as NH_4Cl, monensin or nigericin.[13,14,18] For an accurate FCM-based quantification of surviving cells, an internal standard in the form of inert beads was added to the IT-treated samples prior to FCM-

analysis. The amount of beads that passes the flow cell during analysis, enables the precise calculation of the number of viable cells. Moreover, to decrease the number of false positive/negative cells (*e.g.* due to auto-fluorescence or the extensive disintegration of DNA preventing PI-incorporation) Calc was added as a second viability marker. Only cells being $PI^-/Calc^+$ were considered viable cells.

Figure 2 shows a typical FCM-analysis of Ramos cells 4 days after treatment with a sub-optimal concentration CLB-B-ly/1-ricin A ($5x10^{-11}$ M) in the presence or absence of cytotoxicity enhancer nigericin. The inert beads (10^5/ml) could easily be distinguished from viable as well as non-viable cells based on their relatively small forward scatter and large right angle scatter. As an alternative, beads could be identified by their strong broad-band fluorescence (data not shown). FCM-analyses were stopped when 1,000 beads had passed the flow cell. Comparison of the viable fractions of the control and IT-treated samples revealed a 90-fold increase of efficacy upon addition of nigericin (~0.24 and 2.2 logs of kill with and without nigericin, respectively). Nigericin alone did only marginally effect the viability (<7% reduction of viable cells).

Comparison of FCM-evaluation and clonogenic assays

Conventional clonogenic assays have proven to be a valuable tool for estimation of cell kill. Table 1 presents the reduction in viable cells after incubation different combinations of target-cells and ITs, as determined with FCM-evaluation and clonogenic assays. Both assays revealed comparable results, demonstrating the superiority of the anti-CD22 IT as compared to its anti-CD19 counterpart. Unconjugated MoAbs and ricin A did not influence the viability of any of the target-cells in neither assay (data not shown, n = 3).

Table 1 *Comparison of FCM-evaluation and clonogenic assays.*

	Depletion of target cells (logs)			
	CD19-ricin A		CD22-ricin A	
	clonogenic assay	flow cytometry	clonogenic assay	flow cytometry
Daudi	1.3	1.5	3.0	3.3
KM3	1.3	1.1	2.0	2.7

SDs were less than 10% (n = 3)

Monitoring of cell kill in a heterogeneous sample

Fluorescent labeled Ramos cells were mixed in a 1 to 9 ratio with BM in order to mimic residual leukemic blasts. This mixture was treated with CLB-B-ly/1-ricin A in the presence of NH_4Cl, and assayed for dead and viable cells by FCM-evaluation Figure 3. Their green fluorescence made Ramos cells easily distinguishable from BM cells, including CD19-positive lymphocytes derived from blood admixture. Comparison of the viable fractions of the IT-treated sample and control, demonstrated an approximately 3 logs

of depletion of fluorescent Ramos cells. Omission of cytotoxicity enhancer NH_4Cl reduced the depletion-efficacy to approximately 1 logs (n = 3, data not shown).

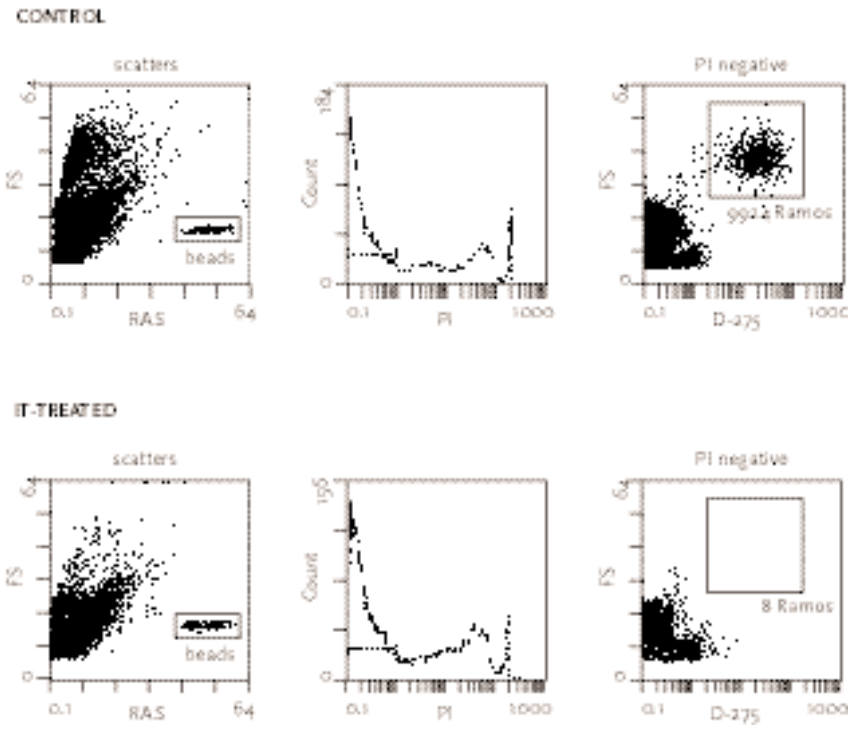

Figure 3 *Monitoring of IT-induced cell kill in bone marrow*

Green fluorescent (D-275) labeled Ramos cells were mixed in a 1:9 ratio with BM cells. The mixture was treated with or without 10^{-8} M anti-CD22 IT (CLB-B-Ly/1-ricin A) in the presence of NH_4Cl (6 mM) at 37°C. Following 24 h, cells were washed and incubated in culture medium for four additional days. Subsequently, cells were stained with PI and evaluated for viability by FCM. Before analysis, a fixed concentration of inert beads (10^5/ml) was added to the samples. Analysis was stopped after 1,000 beads had passed the flow cell. One of three concordant experiments is shown.

DISCUSSION

Viability staining in combination with flow cytometric analysis has been used for diverse applications like monitoring of lymphocyte mediated target-cell lysis and toxicity testing of chemicals.[19-24] The use of this method for evaluation of IT-induced cell kill appeared to be somewhat more complicated. Reason for this is that the toxin-based inhibition of protein synthesis is not accompanied immediately by physical disruption of the cell

membrane. An IT-free incubation period of four days appeared to be necessary for full exposure of IT-toxicity. Moreover, the high potency of ITs (killing up to several logs of target cells) requires a sensitive and precise method for the quantification of residual cells. Simply counting the number of flow cytometric events seems not very adequate, as this parameter is influenced by dead cell particles and fluctuating sampling rates. Therefore, inspired by others, we added a fixed amount of inert beads to the IT-treated sample prior to FCM-analysis.[25] This enabled an accurate quantification of viable cells, which closely correlated with the outcome of conventional clonogenic assays. This is somewhat in contrast with the results obtained by Laurant *et al.*[25] Already in 1986, they described the use of viability staining to evaluate the sensitivity of fresh leukemic cells to ITs. Their assay appeared to be indicative rather than quantitative, though, only roughly qualifying leukemic cells as modestly, intermediate or highly suspectible to IT-treatment. This may be explained by the moment of analysis, directly following IT-treatment, which is before maximal exposure of efficacy.

When evaluating target cell kill in a heterogeneous population as blood or BM, the death/alive analysis needs to be combined with the identification of target-cells (Fig. 3). The use of fluorescent cell specific MoAbs is most convenient, enabling both the viability staining and target-cell identification in one analysis. However, this requires the presence of one or more 'identification-antigens' besides the 'target-antigen' already used for therapy. The latter is inappropriate as it might be modulated or covered by the therapeutic mAb. Moreover, residual malignant cells may be enriched for cells with low expression of target-antigen. As an alternative, target cells may be identified by the PCR-amplification of a cell specific DNA-sequence.[26-28] In that case, viable cells should first be presorted to prevent DNA from IT-eliminated cells contributing to the PCR signal. Based on the specifications of current flow cytometers, a triple-sorted fraction should contain less than one in a million erroneously sorted non-viable cells, equalizing the detection limit of sensitive PCR-assay.

Protein synthesis inhibition is widely used to express the potency of an IT. Estimation of the ID_{50} is relatively easy, fast and independent of *in vitro* clonogenic capacity. Its major disadvantage, though, is that the ID_{50} is hardly influenced by the presence of a minor population of therapy-resistant (potentially tumorogenic) target-cells. The potential relevance of this is demonstrated by observations of Flavel *et al.*, who demonstrated that the combined use of an anti-CD19 and anti-CD38 IT did not reduce the ID_{50} towards Burkitt's lymphoma cell line Ramos, as compared to either IT alone.[29] The survival of Ramos-xenografted mice, in contrast, dramatically increased upon simultaneous administration of both ITs. Apparently, the IT-combination eliminated target-cells with low antigen density which otherwise would escape single IT-treatment. The flow cytometric method is more likely to identify such relatively small cell population, which appears to be of no significance in terms of overall protein synthesis.

From our study, we conclude that the FCM-evaluation of IT-induced cell kill forms a useful addition to the conventional clonogenic assays and protein synthesis inhibition assay. Several studies confirmed that this assay can be used for the monitoring of IT-induced target-cell elimination in freshly derived heterogeneous patient samples.[10,18] As such, this assay may prove a valuable tool to help improve this form of therapy.

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CHAPTER 6

LABORATORY PRODUCTION OF ANTI-CD3 AND ANTI-CD7 RICIN A-IMMUNOTOXINS FOR A CLINICAL PILOT STUDY

Ypke V.J.M. van Oosterhout
J. Liesbeth van Emst
Hans H. Bakker
Frank W.M.B. Preijers
Anton V.M.B. Schattenberg
Dirk J. Ruiter
Sabine Evers
Joop P. Koopman
Theo de Witte

Int J Pharm, in press

ABSTRACT

This report describes the preparation of an immunotoxin-combination, consisting of an anti-CD3 and anti-CD7 monoclonal antibody (MoAb) both conjugated to the A-chain of plant toxin ricin, for the experimental treatment of graft-versus-host disease. MoAbs and toxin were conjugated by conventional biochemical and chromatographic techniques. Raw materials, intermediate and final products were evaluated conform the relevant 'points to consider' of the FDA. Yields, purity and sterility of the two final products were all satisfactory. Preservation of MoAb-affinity and toxin-activity were confirmed in biological assays. The LD_{50}, 25-45 mg immunotoxin-combination/kg mouse, equaled that of similar immunotoxins already in clinical use. Because *in vitro* cross-reactivity screening revealed an unexpected binding of the CD3-MoAb to the esophagus epithelium, human doses immunotoxin-combination were administered to two cynomolgus monkeys. Clinically relevant serum concentrations were obtained without irreversible toxicities occurring. The $T_{1/2}$ varied between ~6-9 hours, the C_{max} ranged from 1.8-3.9 μg/ml. The main side effect was a transient rise of serum creatine kinase. Importantly, neither damage nor binding of the CD3-immunotoxin to the monkey esophagus epithelium could be demonstrated. It was concluded that sufficient material of proper quality and with an acceptable toxicity profile was produced, warranting the evaluation in a clinical pilot-study.

INTRODUCTION

Monoclonal antibodies (MoAbs) conjugated to bacterial or plant-derived toxins (*e.g.* pseudomonas exotoxin or the deglycosylated A-chain of plant toxin ricin), so called immunotoxins, form attractive agents for the selective elimination of disease causing cells (reviewed in 1-3). Within the last two decades, various immunotoxins have been tested in clinical studies for the treatment of life-threatening disorders, including solid

and diffuse tumors and immunological disorders.[4-9] Despite differences in specificity and efficacy, their mechanism of action is basically comparable. The MoAb moiety binds specifically to an antigen expressed on the surface of target cells, wherafter the entire antigen-immunotoxin complex is internalized. Once inside the cell, the toxin-subunit is translocated to the cytosol where it inhibits protein synthesis irreversibly by means of a catalytic reaction culminating in the cell's death. The potency of a given immunotoxin depends in essence on the number of immunotoxin-molecules internalized, and on the intracellular handling following internalization.[10-14] Recently, we demonstrated in laboratory experiments that a combination of two deglycosylated ricin A-chain (dgA) based immunotoxins, directed against lymphocyte differentiation antigens CD3 and CD7, acts synergistically in killing activated T cells.[9] This makes this particular immunotoxin-combination potentially valuable to control certain immunological disorders, like transplantation related rejection and several auto-immune diseases. To explore its therapeutic potential, a clinical pilot study is set up for the treatment of patients with severe graft-versus-host disease, a serious complication of hematological stem cell transplantation initiated by donor-derived T cells.[9] This document describes the semi-large scale laboratory production and quality control of the clinical grade immunotoxin-combination used for the pilot-study.

MATERIALS AND METHODS

Processes, facilities and documentation

The manufacturing processes and the production facilities were designed to minimize the risk of contamination of raw materials, intermediates and the two final products. Prior to the production, the formulae, manufacturing method and specifications of all (intermediate) products were established and documented. The documentation included instructions for operation of equipment, the manufacturing, packaging and storage.

MoAb and dgA

Hybridoma cells producing murine MoAb SPV-T3a (αCD3, IgG2bκ)[15] and WT1 (αCD7, IgG2aλ)[16] were kindly provided by Drs. H. Spits (NKI, Amsterdam, The Netherlands) and W. Tax (UMC St Radboud, Nijmegen, The Netherlands), respectively. From both hybridomas, a master cell bank (MCB) and manufactering working cell bank (MWCB) were prepared. The production of MoAb WT1 was performed at the institutional Central Hematology Laboratory. The production of MoAb SPV-T3a, as well as the protein A purification of both MoAb, was performed by IQ Corporation (Groningen, The Netherlands).

For antibody production, hybridoma WT1 was grown in 162 cm^2 culture flasks in RPMI1640 medium (Flow, Irvine, Scotland) supplemented with 8% fetal calf serum

(FCS) (Integro, Zaandam, The Netherlands), glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator with 5% CO_2 in air at 37°C. Hybridoma SPV-T3a was cultured under the same conditions in RPMI1640 supplemented with 8% FCS, D(+)-glucose (2.5 g/l), sodium hydrogencarbonate (2.0 g/l), L-glutamine (0.4 g/l), gentamycine (0.04 g/l), and HybridoCult supplement (IQ Corporation). Cells were first expanded in log-phase and then cultured for another two weeks without medium changes, resulting in a final viability of about 10%. Subsequently, cell cultures were centrifuged, and the supernatants were filter sterilized (crude harvest) and stored at -20°C (SPV-T3a) or 4°C (WT1). For MoAb purification, the crude harvest was 1:1 diluted with 'bindings-buffer', containing 1.5 M glycine and 3.0 M NaCl (pH 8.9), and applied to a protein A column. Affinity bound MoAbs were eluted with citrate buffer (0.1 M citrate, pH 2.8-3.0) and directly neutralized with 0.1 volume 1M Tris-HCl. Subsequently, purified MoAbs were dialyzed against 0.01 M phosphate buffered saline (pH 7.4) (PBS), filter sterilized and stored at a concentration of approximately 4 mg/ml at 4°C.

Clinical grade deglycosylated ricin A (dgA) was purchased from Inland Laboratories (Austin, TX). The dgA contained the two naturally occurring A-chain forms, of 30 and 32 kDa, found in the seeds of *Ricinus communis*.[17,18] The dgA was stored at a concentration of 3.06 mg/ml in phosphate buffered saline (pH 7.2) containing 50% glycerol at -20°C.

MoAb-dgA conjugation

Preparation of MoAb-dgA conjugates was based on the method described by Ghetie *et al*.[19] Sterile and endotoxin-free solutions were prepared by the Department of Clinical Pharmacy. The preparative chromatographic columns were set up as a closed system except for the inlet and outlet which were positioned in a class 100 laminar flow cabinet. Chromatographic media and columns (all Amersham Pharmacia Biotech, Uppsala, Sweden) were sanitized for every conjugation according to the supplier's 'product support files'. The conjugation procedure started with the derivatization of MoAb with an 8 to 12-fold molar excess of crosslinker 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT)[18] (Pierce, Rockford, IL), and the reduction of dgA with 5 mM DTT. Both reactions were carried out at 37°C for 1 hour. Excess SMPT and DTT were subsequently removed by gel filtration on Sephadex G-25 (XK100/50 column) equilibrated with PBS containing 0.05% Tween-20 (PBST). Average MoAb-MPT loading was determined spectrophotometrically, as described elsewhere.[20] For the actual conjugation reaction, MoAb-MPT was then mixed with a 2.5-fold molar excess of freshly reduced dgA-SH. Subsequently, 0.2 volume 85% glycerol was added and the conjugation mixture was gently stirred and the reaction was allowed to proceed at 20-25°C for 48 hours. The resulting crude conjugate was purified by affinity chromatography on Blue-Sepharose (XK50/60 column) for removal of unconjugated MoAb (The Sepharose-conjugated Cybacron Blue has a high affinity for dgA). In case of WT1-dgA,

the Blue-Sepharose was equilibrated with PBST. For SPV-T3a-dgA, the Na^+ concentration of the reaction mixture and loading buffer had to be adjusted to 300 mM in order to prevent affinity-binding of unconjugated SPV-T3a. Affinity-bound dgA and MoAb-dgA conjugates were eluted with PBST containing 1M NaCl. High molecular weight material and free dgA were subsequently removed by size exclusion chromatography on Sephacryl S-300 HR (K100/100 column) equilibrated with PBST (formulation buffer). The resulting immunotoxins were concentrated to approximately 1 mg/ml and stored as infusion concentrates in sterile 5 ml glass vials at -20°C at the Department of Clinical Pharmacy. The immunotoxin-combination is prepared directly for use by diluting equal amounts (w/w) of SPV-T3a-dgA and WT1-dgA in formulation buffer.

Immunotoxin composition and biological activity

The composition of purified MoAb-dgA conjugates was analyzed by nonreducing SDS-PAGE using 4-15% gradient gels (PhastSystem, Amersham Pharmacia Biotech). The ratio of different reaction products was determined by densitometric scanning of the separated product bands of Coommassie blue stained gels. Samples containing a 1:1 mixture of unconjugated dgA and MoAb were used for compensation of variance in dye-uptake between both proteins.

Binding capacity of the MoAb before and after conjugation to dgA was examined by a standard flowcytometric titration assay using an antigen positive cell line and a fluorescent labeled goat-anti-mouse antibody to detect cell bound MoAb. Preservation of Ricin A toxicity was determined by measurement of protein synthesis inhibition in a cell free reticulocyte assay.[21]

These analyses were periodically repeated and compared to baseline values to monitor the chemical stability and biological activity of the infusion concentrates during storage.

Sterility, endotoxins and adventitious agents

The FCS added to the culture medium was derived from healthy calves housed in a BSE-free environment, and tested negative for Bovine Diarrhoea virus, Infectious Bovine Rhinotracheitis, Parainfluenza and Bovine Polyoma virus. The MWCB of SPV-T3a and WT1 were tested for bacterial and fungal contamination and for mycoplasm. An 'extended S^+L^- focus assay' and 'extended XC-plaque assay' was performed by Inveresk Research (Tranent, Scotland) for detection of xenotropic and ecotropic retroviruses, respectively. Species specific viruses were tested by the institutional Department of Virology using a mouse antibody production test (MAP-assay), detecting 16 different exogenous mouse viruses including sendai virus, reovirus type 3, Mouse rotavirus (EDIM), hantaan virus, and lymphocytic choriomeningitis virus. The presence of adventitious viruses was tested by the Department of Virology using 8 different indicator cell lines (including 3 human) and by inoculation of embryonated chicken eggs. Purified MoAb were tested for endotoxins, sterility and mycoplasma. The final immunotoxins were tested for the presence of hybridoma DNA by Inveresk Research. The leak-

age of affinity chromatography media was assayed using commercial enzyme immunoassays according to the manufacturer's instructions: Protein A using a kit from Cygnus Technologies (Wrentheam, MA, USA), Cybacron Blue with a kit from Affinity Chromatography Ltd (Girton, Cambridge, UK). The stored infusion concentrates were tested for endotoxins, sterility and mycoplasma.

Cross reactivity with human tissues

Potential cross-reactivity of MoAb SPV-T3a and WT1 was tested by immunostaining of the following panel of quickly frozen human tissue sections derived from the tissue bank at the Department of Pathology (# donors): heart (3), lymph node (3), spleen (3), lung (3), esophagus (3), stomach (3), ileum (3), colon (3), liver (3), pancreas (3), testis (3), prostate (3), bladder (3), pyelum (3), kidney (3), ovary (3), Fallopian tube (2), uterus (3), breast (3), cerebrum (3), cerebellum (3), spinal cord (1), skin (3), striated muscle (3), thyroid (3), parathyroid (2), adrenal gland (3), pituitary (2), eyeball (1). Reactivity against hematopoietic cell lines and stem cells has been tested extensively during the initial characterization of the MoAbs. Potential cross-reactivity against embryonic/fetal tissues has not been determined yet since pregnant women are no candidates for allogeneic stem cell transplantation.

In vitro dissociation in human plasma

Dissociation of MoAb and dgA during incubation in human plasma at 37°C was monitored using enzyme immunoassays which detect intact MoAb-dgA conjugates.[9]

LD_{50} mice

All animal experiments were performed by personnel of the Central University Animal Laboratory in accordance with the 'Principles of Laboratory Animal Care' (NIH publication #85-23, revised 1985). The LD_{50} experiment was performed with male BALB/C mice of eight weeks weighing ~17 g, as described by Weil.[22] Four doses were administered: 96, 193, 385 and 770 μg immunotoxin-combination per mouse, in a final volume of 200 μl. Administration was intravenously through one of the tail veins. Upon administration, mice were weighed daily and followed for survival for two weeks. The control group was injected with formulation buffer only.

Administration to cynomolgus monkeys

The monkeys received two doses of immunotoxin-combination, administered 48 hours apart as 100 ml infusions over a period of 4 hours. Prior to infusion, the monkeys were sedated (ketamine 10 mg/kg, atropine 0,5 mg) and subsequently anesthetized (ratio oxygen and nitrous oxide 1:2, enflurane 1,5%). The immunotoxin-combination was administered via a subclavian (first infusion) or femoral (second infusion) central venous catheter. For detection of any esophagus toxicity, endoscopy was performed on day -7, 4 and 9 (day 1 being the first infusion day). During endoscopy, biopsies were taken for microscopic analysis of potential tissue damage. Moreover, biopsy derived tissue was stained with a peroxidase-labeled anti-mouse-IgG2b antibody for detection of

SPV-T3a-dgA. Tissue pre-incubated *in vitro* with saturating amounts of SPV-T3a-dgA served as positive control. In addition, both monkeys underwent regular physical examinations and blood chemistries, complete blood counts and leukocyte differentials were obtained for general safety assessment. One of the monkeys (receiving 0.1 and 0.2 mg/kg immunotoxin-combination) suffered from diabetes mellitus, but showed no physical disorders which were expected to influence the outcome of the experiment.

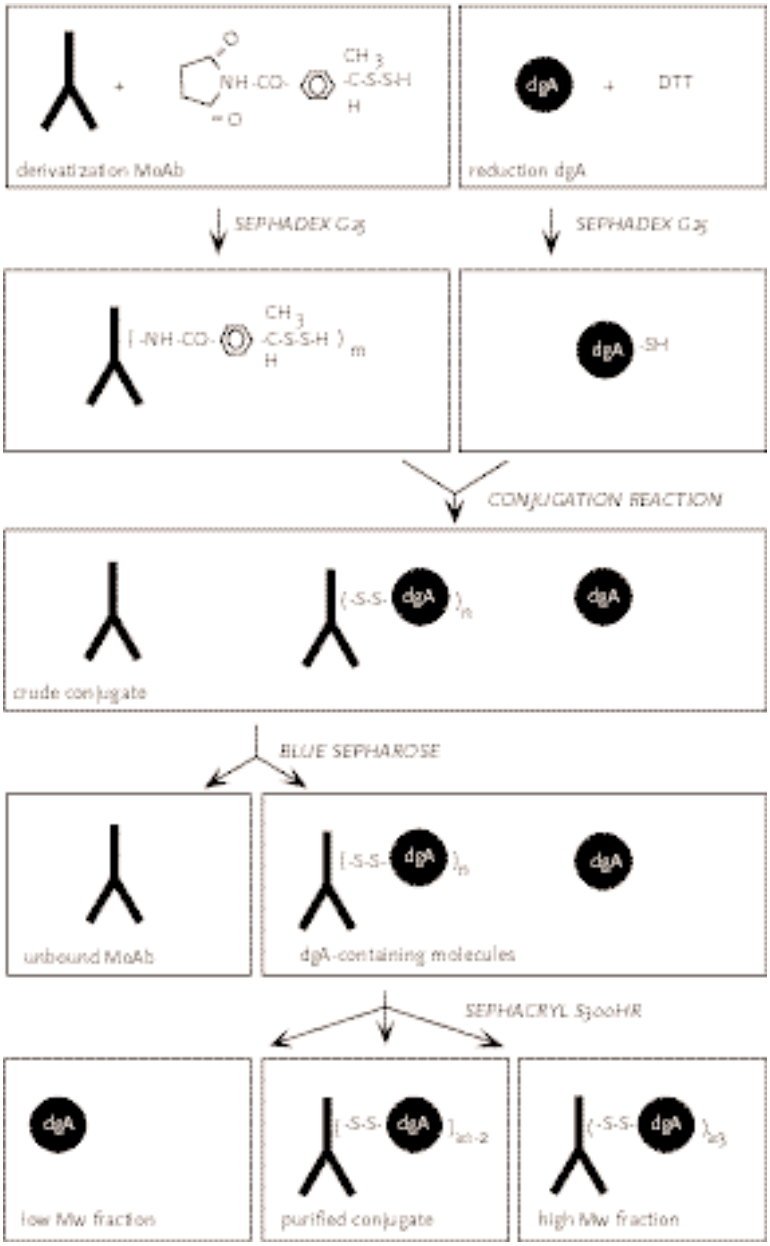

Figure 1 *Immunotoxin preparation*

MoAb and dgA are derivatized with SMPT (m-fold, with m = 0, 1, 2, ...) and reduced with DTT, respectively. The purified reaction mixtures (Sephadex G25) are mixed and incubated at room temperature for 48 hours. The resulting crude conjugate, containing protein aggregates, MoAb conjugated to dgA (n-fold, with n ≤ m), and non-conjugated molecules, are then separated by BlueSepharose-affinity chromatography and S300-size exclusion chromatography.

RESULTS

Immunotoxin production

The crude harvest of SPV-T3a and WT1 contained approximately 50 μg/ml MoAb. The recovery following Protein A purification was ~85%, about 1 g of purified SPV-T3a and WT1 were produced. The subsequent conjugation process is schematically depicted in figure 1. To reduce the risk of losing all material by a technical failure, MoAbs were divided in two fractions which were conjugated separately to dgA and subsequently partly purified by BlueSepharose. The MoAb-dgA containing BlueSepharose eluates of each MoAb were then pooled and applied as one fraction to the Sephacryl S-300 column. The final MoAb-recovery following conjugation was 34% and 26% for SPV-T3a and WT1, respectively. Figure 2 displays a representative non-reducing SDS-PAGE analysis of an SPV-T3a-dgA conjugation.

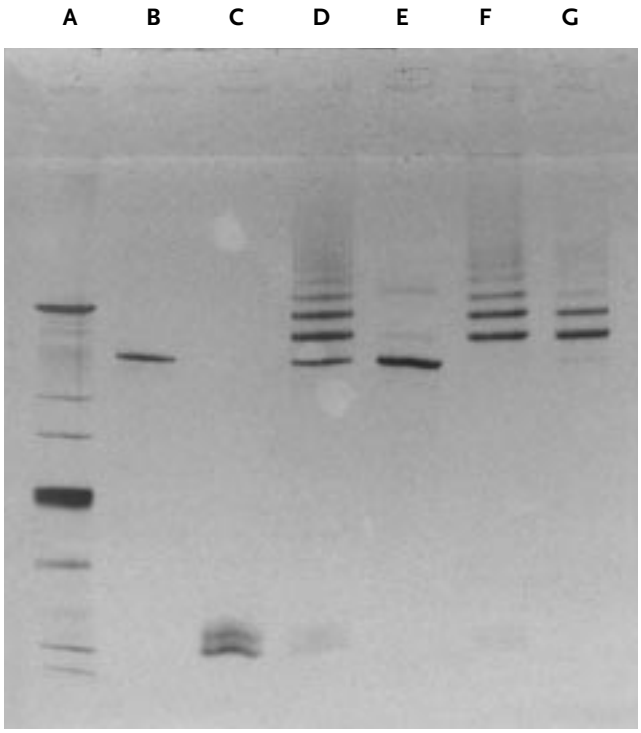

Figure 2 *SDS-PAGE analysis of SPV-T3a-dgA conjugation*

A: high molecular weight marker; B: SPV-T3a (IgG2a); C: reduced dgA; D: crude conjugate; E: run-through fraction BlueSepharose; F: high-affinity eluate BlueSepharose; G: pooled fractions of Sephacryl S300HR eluate containing SPV-T3a-dgA.

Table 1 summarizes the main characteristics of the final products, the left column displaying the proposed release criteria. Ideally, the final product consists entirely of MoAb conjugated to one or two dgA molecules. In practice, the final product contains some unconjugated MoAb, and MoAb bound to three or more dgA molecules, as well. The composition of WT1-dgA did not strictly meet the (arbitrary) release criteria as for its relatively high amount of free MoAb (23% instead of <10%). However, the acceptance of WT1-dgA in its present form was justified by the absence of 'contamination' with MoAb conjugated to three or more dgA-molecules, as well as by the high preservation of antigen-binding activity (90%) and effective *in vitro* killing capacity.[9] Another point of

Table 1 *Product specifications*

	Proposed Release Criteria	SPV-T3a-dgA (CD3)	WT1-dgA (CD7)
MoAb-Isoform	IgG2bκ/IgG2aλ	IgG2bκ	IgG2aλ
Composition:			
$MoAb(dgA)_{\geq 3}$	<10%	6%	0%
$MoAb(dgA)_{1\text{-}2}$	>80%	88%	78%
MoAb	<10%	6%	23%
dgA	<3%	<1% (detection limit)	<1%
Sterility	Sterile	Sterile	Sterile
Endotoxins (EU/mg protein)	< 20	< 0.8	< 0.7
DNA content (pg/mg protein)	< 5	< 11	< 9
Protein A (ng/mg protein)	< 10	1.1 ± 0.3	2.7 ± 0.8
Cybacron Blue (nM)	< 10 (detection limit)	< 10	< 10
A-chain activity (% native dgA)	> 75	84	91
Binding activity (% native MoAb)	> 50	70	90
Dissociation in vitro (% in 24h)	~15	10	10
In vitro cross reactivity	Absent. If not, relevant animal studies should follow	Esophagus epithelium (++) Smooth muscle cells (+/-)	Kupfer cells (+/-)
LD_{50} in mice (μg/g)	~14 μg/g mouse	25-45 μg/g mouse for the IT-combination	
Toxicity for cynomolgus monkey	No severe irreversible toxicities	Reversible toxicity to skeletal muscles after 0.1-0.25 mg/kg IT-combination	

attention was the presence of genomic DNA. The IT-combination contains <10 pg DNA/mg protein, being the detection limit of the assay. The relevant FDA's 'Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use' suggests that, whenever possible, the final product contains no more than 100 pg cellular DNA per dose. This implies that total human doses of 10 mg and higher (equalizing ~5-7 mg/m^2) acquire additional testing (*e.g.* with concentrated immunotoxin to lower the detection limit).

So far, the repeated testing for composition, MoAb-binding and dgA-activity demonstrated stability of both SPV-T3a-dgA and WT1-dgA at -20°C for a minimum period of three years.

Cross reactivity with human tissues

MoAb SPV-T3a showed two unexpected cross-reactivities: against (1) smooth muscle cells, and (2) basal epithelial cells of the esophagus. The staining of smooth muscle cells was weak (+/-) but consistently found throughout various tissues. The diffuse staining pattern suggested a cytoplasmatic binding of physically disrupted cells. This is supported by the observation that SPV-T3a-dgA (10^{-8}M for 24 hours) appeared to be non-toxic to cultured intact human leptomeningial smooth muscle cells (data not shown). The staining intensity of SPV-T3a with the esophagus-basal epithelium was as strong (++) as observed with positive lymphocytes, and appeared to be characteristic for SPV-T3a since it was not observed with any of the other CD3 MoAbs tested (OKT3, UCHT and WT32). The *in vivo* consequence of this cross reactivity will depend on the local concentration SPV-T3a-dgA, and whether SPV-T3a-dgA will be internalized (a prerequisite for toxicity). To gain more insight in these parameters, it was decided to administer the immuntoxin-combination to cynomolgus monkeys, demonstrating *in vitro* cross-reactivity of comparable intensity and localization.

The screening with MoAb WT1 revealed a weak staining (+/-) of some Kupffer cells located in the liver sinus wall. This staining pattern was not observed during previous preclinical tissue screenings performed with WT1.[23] No further unexpected cross-reactivities were observed.

LD50 mice

The LD_{50} in mice was determined for comparison with reference immunotoxin RFB4-dgA, a comparable immunotoxin already tested in FDA-approved phase I/II trials (having a LD_{50} of 14 mg/kg).[24] Three of the four mice treated with the highest dose immunotoxin-combination (770 μg/mouse) died within the follow-up period of two weeks. One mouse already died during injection, probably due to an administration-related toxicity (dgA-related deaths are normally first seen after 1-2 days). All mice treated with 385 μg immunotoxin-combination or less survived. Based on the average initial weight, this implies an LD_{50} of 25 - 45 mg/kg mouse.

Apart from the survival score, mice were daily weighed and observed for behavior. Administration of IT combination caused weight loss in all groups, which was most pronounced after 9 days (Figure 3). The extent and duration correlated well with the dosage. Besides weight-loss, mice treated with 770 or 385 μg IT-combination demonstrated lethargy, lack of appetite and a neglected fur. The mice treated with the two lower doses could not be distinguished visually from the control group.

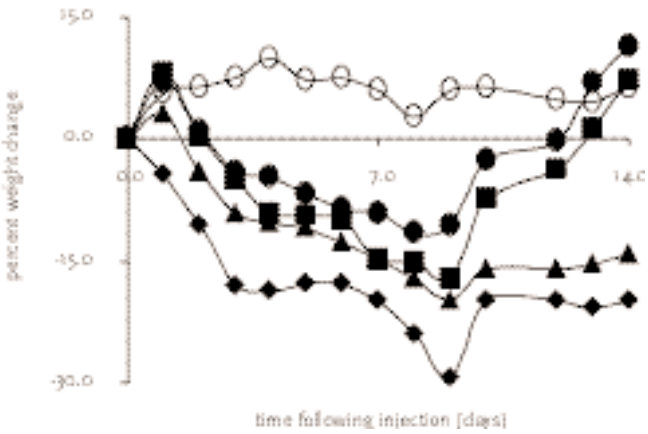

Figure 3 *Average weight-changes of immunotoxin treated mice*

Mice were treated with formulation buffer (❍) or with 96 μg (●), 193 μg (■), 385 μg (▲) or 770 (g immunotoxine-combination/mouse (◆). Upon administration, mice were weighed daily for two weeks.

Administration of the immunotoxin-combination to cynomolgus monkeys

The imunotoxin-combination was administered to two male cynomolgus monkeys. As the administration was performed under general anesthesia, the animals had to be deprived of nutrition before each infusion. Since this may negatively impact their physical condition, the administration was restricted to two doses at a 48-hour interval (the patients in the pilot-study were supposed to receive four doses at the same interval). This decision was justified by observations of Amlot *et al*. who demonstrated that the toxicity of a dgA-based immunotoxin mainly depends on the height of the individual doses and not on the number of doses administered.[24] Both monkeys received two of the higher individual doses of the proposed human dose escalation scheme (ranging from 2 to 10 mg/m^2), translated to mg/kg. One monkey (about 12 years and 7.6 kg) received 0.1 and 0.2 mg/kg, the other monkey (4 years and 4.1 kg), received 0.2 and 0.25 mg/kg (equalizing human doses of 4, 8 and 10 mg/m^2). In human, the immunotoxin-combination will be given as a 100 ml infusion over 4 hours. For the monkeys, this was changed to 20 ml administered in 1 hour because of their smaller blood volume.

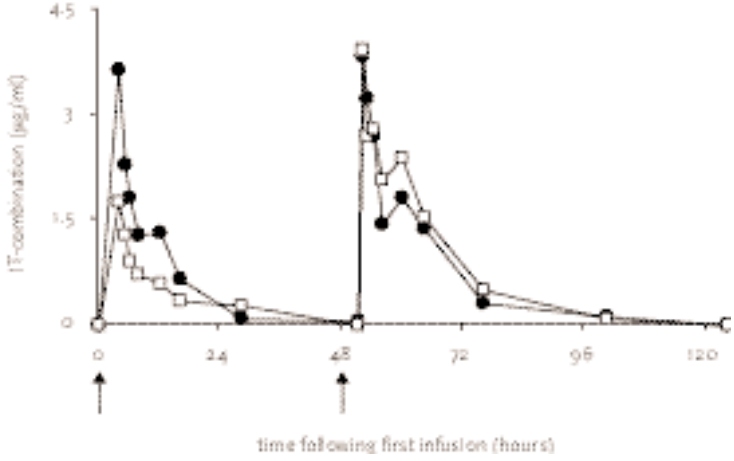

Figure 4 *Immunotoxin-combination plasma concentrations in cynomolgus monkeys*

Two male cynomolgus monkeys were each given two doses immunotoxin-combination with an interval of 48 hours (arrows beneath the x-axis). One monkey received 0.1 and 0.2 mg/kg (❑), the other 0.2 and 0.25 mg/kg (●).

Figure 4 shows the plasma concentrations of the immunotoxin-combination. The clearance curves best fitted a two-compartment model for both SPV-T3a-dgA and WT1-dgA individually and given in combination. The pharmacokinetic parameters are listed in

Table 2 *Pharmacokinetic parameters (± SD)*[a]

Monkey (dose, mg/kg)	Immunotoxin	$T_{1/2}\beta$(h)	AUC (µg·h/ml)	Cl (ml/h)	V_d (ml)	C_{max} (ng/ml)
A	SPV-T3a-dgA	9.1 ± 0.6	9.4 ± 1.6	26.5 ± 4.5	347.3 ± 29.1	1,830
(0.1 and 0.2)	WT1-dgA	9.4 ± 1.6	12.8 ± 1.7	19.6 ± 2.6	265.8 ± 17.5	2,090
	Combination	9.3 ± 1.7	22. 3 ± 3.1	4.5 ± 0.6	60.3 ± 4.2	3,920
B	SPV-T3a-dgA	9.3 ± 2.0	23.5 ± 3.8	8.5 ± 1.4	113.6 ± 9.2	2,280
(0.2 and 0.25)	WT1-dgA	2.2 ± 0.2	4.7 ± 0.3	42.9 ± 3.1	133.7 ± 7.4	1,450
	Combination	5.7 ± 1.0	24.8 ± 3.3	8.1 ± 1.1	65.8 ± 4.6	3,840

[a]*Abbreviations: $T_{1/2}\beta$, plasma half-life; AUC, area under the concentration versus time curve; Cl, clearance; V_d, volume of distribution; and C_{max}, maximum plasma concentration.*

Table 2. The $T_{1/2}$ of the immunotoxin-combination was 9.3 and 5.7 hours in monkeys A en B, respectively. Notably the $T_{1/2}$ of SPV-T3a and WT1 in 'monkey B' differed considerably, being approximately 9 and 2 hours, respectively. This may be explained by the capture of WT1-dgA by the CD7-antigen expressed on T and NK cells (virtually all these cells being $CD7^+$ in 'monkey B'). 'Monkey A' with only ~30% of its T/NK cells being

$CD7^+$, demonstrated equal half lives for SPV-T3a-dgA and WT1-dgA (~9 hours). It should be noted that SPV-T3a-dgA is not influenced by expression of the CD3-antigen as SPV-T3a does not bind 'monkey-CD3'. Peak plasma levels were attained directly following each infusion and decreased (nearly) to baseline level in about 48 hours. The C_{max} of the immunotoxin-combination ranged from 1.8 to 3.9 μg/ml and strongly correlated with the dose administered. In general, plasma levels of ~1.8 μg/ml (10^{-8} M) are considered therapeutic concentrations.[25] In both monkeys, these plasma concentrations were maintained for 8-10 hours following the second infusion.

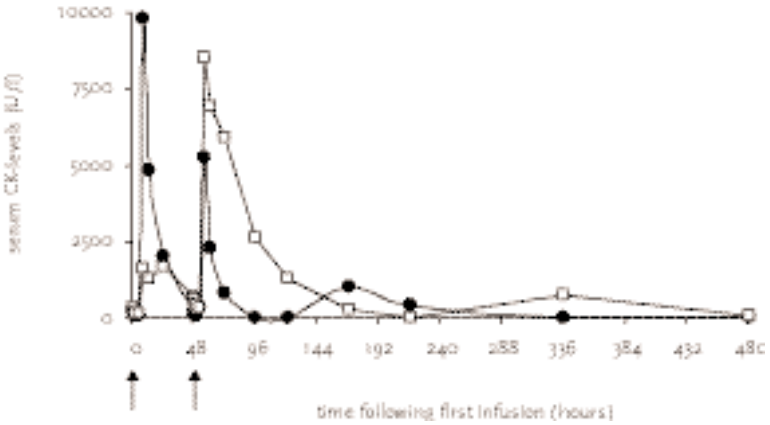

Figure 5 *Serum CK-levels in cynomolgus monkeys*

Following administration of the immunotoxin-combination, both cynomolgus monkeys demonstrated a transient rise of serum CK-levels. One monkey received 0.1 and 0.2 mg/kg (❑), the other 0.2 and 0.25 mg/kg (●) immuntoxin-combination, separated by a 48-hour interval.

Regarding the cross-reactivity of SPV-T3a, neither macroscopic nor microscopic examinations revealed any evidence of toxicity towards the esophagus. Moreover, immunostaining of biopsy derived tissue could not demonstrate any *in vivo* binding of SPV-T3a-dgA to the esophagus epithelium. Blood chemistry analyses revealed a transient drug-related toxicity. Each administration was followed by a sharp increase in serum creatine kinase (CK), reaching a maximum at 7 hours after infusion and normalizing to baseline within 2-7 days (Figure 5). Following the second peak, both monkeys demonstrated a third, more modest, rise of CK-levels that might be explained by a secondary release of dgA. The increase in CK levels is indicative for destruction of striated muscles and/or heart muscle. Damage of the heart muscle could be excluded by measurement of heart-muscle isomer CKMB. In accordance with the destruction of striated

muscle cells, a concomitant (modest) increase in LD, ASAT and ALAT serum levels could be noted. Four weeks after completion of the study all biochemical parameters had returned to baseline levels. Complete blood counts and leukocyte differentials displayed a normal distribution. Immediately after the infusions, both monkeys seemed a bit weakened. Aside from the general anesthesia, this may have resulted from the destruction of striated muscle tissue. One day after the second dose, both monkeys ate almost as normal again. Within a week, both monkeys functioned as before treatment.

DISCUSSION

This report describes the laboratory preparation and control of two dgA-based immunotoxins that are currently under evaluation in a clinical pilot-study. The design of this study was strongly inspired by earlier reports on RFB4-dgA, a comparable immunotoxin for the treatment of Non-Hodgkin's lymphoma.[24,26] The murine LD50 of the immunotoxin-combination compared favorably with that of RFB4-dgA (25-45 mg and 14 mg per kg mouse, respectively).[19] Moreover, the monkey experiment demonstrated pharmacokinetics resembling those previously observed with RFB4-dgA and comparable immunotoxins in man. Importantly, clinically significant plasma concentrations were reached for several hours without being accompanied by acute irreversible toxicities. With respect to the potential cross-reactivity of SPV-T3a-dgA, it was particularly of interest that no binding or toxicity to the esophagus was observed. The main toxicity observed was a clear but transient rise in CK-levels. This is in accordance with myalgias, sometimes associated with rhabdomyolysis, being the second dose limiting toxicity associated with dgA-immunotoxins in man (after vascular leak syndrome, VLS).[25] Consistent with the human studies, this toxicity resolved upon ending the administration of immunotoxin. It should be noted, though, that the animal studies do not eliminate all uncertainties with regard to clinical administration of the immunotoxin-combination. The esophagus of patients with severe graft-versus-host disease may be more accessible for SPV-T3a-dgA (*e.g.* due to disease-associated lesions) than those of healthy cynomolgus monkeys. Another concern is that monkeys are hardly vulnerable for dgA-induced VLS, the main toxicity reported in man. However, as these issues can not be clarified by animal studies, the current data were considered maximally supportive for performing the clinical pilot-study.

So far, the preliminary results of the clinical pilot-study are very encouraging and do warrant the set up of multi-center Phase I/II studies. Consequently, in addition to a further scaling-up of production, product-specifications will probably have to meet stricter criteria. A point for improvement forms the reduction of the relatively high amount of unconjugated WT1. Moreover, stricter guidelines might ask for future MoAb productions to be performed in serum-free medium. This inevitably will result in repeated

hybridoma cell banking and subsequent virus testing, as changes in growing conditions might provoke the expression of different latent viruses. As an additional requirement, the actual viral removing capacity of the production process will need to be demonstrated by virus spiking using a scale-down model. Though the FDA foresees in the human testing of (modified) MoAb combinations, it is anticipated that the required animal toxicity studies (*e.g.* acute/subchronic toxicity and reproduction toxicity studies) will have to be performed with both immunotoxins separately.

Notwithstanding the foreseen adaptations, the current batch is very well suited for its intended use: the evaluation in a clinical pilot study for the treatment of a life-threatening disease. As such, the described production forms a stepping stone towards the future productions as required for the actual registration process. It provided sufficient quantities of proper quality to enable the clinical evaluation in an academic setting. Especially for niche indications like severe graft-versus-host disease, some clinical proof of concept appears a prerequisite for further development by the pharmaceutical industry. The in house production and clinical evaluation of biological therapeutics by academic centers might therefore contribute increasingly to the development of new drugs within this area.

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CHAPTER 7

A COMBINATION OF ANTI-CD3 AND ANTI-CD7 RICIN A-IMMUNOTOXINS FOR THE IN VIVO TREATMENT OF ACUTE GRAFT-VERSUS-HOST DISEASE

Ypke V.J.M. van Oosterhout
J. Liesbeth van Emst
Anton V.M.B. Schattenberg
Wil J.M. Tax
Dirk J. Ruiter
Hergen Spits
Fokke M. Nagengast
Roos Masereeuw
Sabine Evers
Theo de Witte
Frank W.M.B. Preijers

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ABSTRACT

This study evaluated the anti-graft versus host disease (GVHD) potential of a combination of immunotoxins (ITs), consisting of a murine CD3 (SPV-T3a) and CD7 (WT1) monoclonal antibody both conjugated to deglycosylated ricin A. *In vitro* efficacy data demonstrated that these IT act synergistically, resulting in an approximately 99% elimination of activated T cells at 10^{-8}M (about 1.8 μg/ml). Because most natural killer (NK) cells are $CD7^+$, NK activity was inhibited as well. Apart from the killing mediated by ricin A, binding of SPV-T3a by itself impaired *in vitro* cytotoxic T-cell cytototoxicity. Flow cytometric analysis revealed that this was due to both modulation of the CD3/T-cell receptor complex, and activation-induced cell death. These results warranted evaluation of the IT-combination in patients with refractory acute GVHD in an ongoing pilot study. So far, 4 patients have been treated with 3 to 4 infusions of 2 or 4 mg/m^2 IT-combination, administered intravenously at 48-hour intervals. The $T_{1/2}$ was 6.7 hours, and peak serum levels ranged from 258 to 3,210 ng/ml. Drug-associated side effects were restricted to limited edema, fever, and a modest rise of creatine kinase levels. One patient developed low-titer antibodies against ricin A. Infusions were associated with an immediate drop of circulating T cells, followed by a more gradual but continuing elimination of T/NK cells. One patient mounted an extensive CD8 T-cell response directly following treatment, not accompanied with aggravating GVHD. Two patients showed nearly complete remission of GVHD, despite unresponsiveness to the extensive pretreatment. These findings justify further investigation of the IT-combination for treatment of diseases mediated by T cells.

INTRODUCTION

Stem cell transplantation forms a widely accepted method for restoration of normal hematopoiesis of patients treated for a hematological malignancy or otherwise suffer-

ing from a defective hematopoietic or immunologic system. For a successful engraftment, a minimal number of donor T cells in the graft appears to be a prerequisite. The underlying mechanism by which these cells promote engraftment is not fully understood, but probably includes the creation of an immunologically tolerogenic environment by eliminating the remainder of the patient's immune system.

In treatment of a malignancy, the cotransplantation of donor T cells confers additional benefit because they contribute to the so-called graft-versus-leukemia (GVL) effect, which involves the elimination of residual malignant cells.[1] The basis of GVL forms the recognition by donor T cells of (minor or major) histocompatibility antigens expressed by the malignant recipient cells. Unfortunately, this antigenic disparity may also lead to graft versus host disease (GVHD), a major cause of morbidity and mortality after allogeneic stem cell transplantation.[2] GVHD is thought to be initiated by alloactivation of donor T cells resulting in the production of cytokines (interleukin [IL]-2 and interferon [INF]-γ) which in turn activate additional effector cells, like monocytes, macrophages, and natural killer (NK) cells, to produce inflammatory proteins (IL-1, tumor necrosis factor [TNF]-α, and IL-6).[3] This may result in serious direct and indirect cytotoxicity to epithelial cells of skin, liver, and gut.

When prophylaxis has failed (typically cyclosporine, often combined with methothrexate), severe GVHD is usually treated with low-dose corticosteroids. If the reaction progresses, the dose may be increased (up to levels of 10-20 mg/kg/day) or, alternatively, polyclonal antithymocyte/antilymphocyte globulin (ATG/ALG) or experimental immunosupressive drugs may be applied. An example of such an experimental reagent is Xomazyme-CD5 Plus, a murine CD5 monoclonal antibody (MoAb) conjugated to the A-chain of the phytolectin ricin. Especially in the initial reports, Xomazyme-CD5 Plus demonstrated substantial efficacy in treating steroid-resistant acute GVHD.[4,5] In more recent comparative trails, Xomazyme-CD5 Plus was not more effective than high dose corticosteroids or ATG.[6,7] Encouraged by its initial success, we developed a therapy based on the use of a combination of 2 anti-T-cell immunotoxins (IT): murine MoAb SPV-T3a (CD3) and WT1 (CD7), both conjugated to deglycosylated ricin A (dgA). In this article, we present the preclinical efficacy data and preliminary clinical results, both of wich suggest that this IT-combination has the potential for helping to control severe acute GVHD .

MATERIALS AND METHODS

Immunotoxins

The "IT-combination" as referred to in this article, consists of a 1:1 mixture (w/w) of murine MoAb SPV-T3a (CD3)[8] and WT1 (CD7)[9,10] both conjugated to deglycosylated ricin A (dgA) (Inland, Austin, TX) using the SMPT-cross-linker (Pierce, Rockford, IL),

according to Ghetie *et al*.[11] The preparation and validation were supervised by the institutional Department of Clinical Pharmacy. The major characteristics of the IT are summarized in Table 1; details will be reported elsewhere (manuscript in preparation). Murine MoAb UPC-10 (IgG2a) and MOPC-141 (IgG2b) (both Sigma, St. Louis, MO) conjugated to dgA served as isotype-matched irrelevant controls for the *in vitro* experiments. Noteworthy, *in vitro* tissue screening unveiled a cross-reactivity of MoAb SPV-T3a with the basal epithelium of the human esophagus. To investigate potential *in vivo* consequences, the IT-combination was administered to two cynomolgus monkeys sharing the same cross reactivity. Subsequent esophagus biopsies revealed no toxicity towards the esophagus epithelium, nor could any localization of SPV-T3a-dgA be detected. Both monkeys demonstrated a transient rise of creatine kinase (CK)-levels upon administration of the IT-combination.

Table 1 *IT characteristics*

	SPV-T3a-dgA (CD3)		WT1-dgA (CD7)	
MoAb	murine IgG2bκ		murine IgG2aλ	
Composition (SDS-PAGE)	$MoAb(dgA)_{\geq 3}$	6%	$MoAb(dgA)_{\geq 3}$	0%
	$MoAb(dgA)_{1\text{-}2}$	88%	$MoAb(dgA)_{1\text{-}2}$	78%
	MoAb	6%	MoAb	23%
	dgA	0%	dgA	0%
Sterility	Sterile		Sterile	
Endotoxins (EU/mg protein)	< 0.8		< 0.7	
DNA content (pg/mg protein)	< 11		< 9	
Protein A (ng/mg protein)	1.1 ± 0.3		2.7 ± 0.8	
Cybacron Blue (nM)	< 10		< 10	
A-chain activity (% native dgA)	84		91	
Binding activity (% native MoAb)	70		90	
Dissociation in human plasma at 37°C (% in 24h)	10		10	
In vitro cross reactivity	Esophagus epithelium (++) Smooth muscle cells (+/-)		Kupfer cells (+/-)	
LD_{50}	25-45 μg/g mouse for the IT-combination			
Toxicity cynomolgus monkey	Reversable increase of CK-levels upon administration of human doses IT-combination (0.1-0.25 mg/kg)			

Peripheral blood mononuclear cells (PBMC)

PBMC were isolated from peripheral blood by Ficoll-Hypaque density centrifugation (Pharmacia, Uppsala, Sweden), and cultured in Iscove's medium (Flow, Irvine, Scotland) supplemented with 10% heat-inactivated Pooled Human Serum (PHS), penicillin (100 U/ml) and streptomycin (100 μg/ml). For generation of PHA-activated T cells, PBMC were preincubated with 40 μg/ml phytohaemagglutinin (PHA-HA15) (Murex, Dartford, UK) for 48 hours.

Flow cytometric quantification of in vitro cell kill

Nonactivated as well as PHA-activated PBMC (10^6/ml) were incubated in triplicate with varying concentrations of IT (10^{-13}-10^{-8} M) at 37°C for 24 hours. Following treatment, cells were washed and cultured in the presence of PHA (40 μg/ml) for an additional four days to enable full exposure of IT toxicity. Subsequently, cells were labeled with propidium iodine (PI) (Molecular Probes, Junction City, OR) and calcein AM (Calc) (Molecular Probes) (both: 2 μg/ml for 30 minutes at room temperature), and analyzed on a flow cytometer (Coulter Epics Elite, Hialeah, FL). Cells being PI^- and $Calc^+$ were referred to as viable cells. Prior to FCM analysis, a fixed amount of inert beads (Flow-Count fluorospheres, Coulter) was added to each sample ($1x10^4$/ml) to enable the quantification of surviving cells.

Reduction of CTL toxicity by SPV-T3a

Cytotoxic T cell (CTL) toxicity was assayed *in vitro* using an EBNA3C12-reactive CTL clone. The CTL clone was incubated with MoAb SPV-T3a (10^{-8} M), or isotype matched control MoAb MOPC-141, at 37°C for 24 hours. Subsequently, cells were washed and cultured in culture medium for another 72 hours. Remaining cytolytic activity was assayed with an autologous Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line (EBV-LCL), labeled with 100 μCi ^{51}Cr (Amersham, Bucks, UK) at 37°C for 2 hours. Labeled EBV-LCL were plated in triplicate (10^3/well) in V-bottom microtiter plates (Greiner, Fickenhausen, Germany) and saturated with endogenous EBNA3C (5 μM for 1 hour at 37°C) to enhance T-cell receptor (TCR)-mediated lysis. Subsequently, varying numbers of MoAb-treated CTL cells were added to each well in a final volume of 150 μl culture medium. Plates were centrifuged (50*g*, 1 minute) and further incubated at 37°C. After 4 hours, 100 μl supernatant was collected from each well and counted in a gamma counter. Specific lysis was expressed as percentage maximal lysis by detergent, both corrected for spontaneous ^{51}Cr-release.

Modulation of the CD3-antigen by SPV-T3a

Surface CD3 expression of SPV-T3a-treated CTL was determined by indirect fluorescence staining with a saturating amount of SPV-T3a (10 μg/ml, 4°C 30 minutes), followed by a fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat-anti-mouse IgG (American Qualex International, La Mirada, CA). CD3-antigen still bound by SPV-T3a used for treatment was identified by staining with the FITC-conjugated antibody only. Expression was indicated as percentage relative to untreated control cells.

In vitro reduction of NK-activity.

The PBMC (10^6/ml) were incubated with 10^{-8}M MoAb or IT for 24 hours. Subsequently, cells were washed and cultured for 3 additional days without MoAb/IT (to enable full exposure of toxicity). PBMC were serially diluted in 96-well U-bottomed plates, and a fixed concentration of ^{51}Cr-labeled K562 cells was added (10^4/well) to yield effector/target ratios of 10:1 to 0.37:1 in a final volume of 150 μl culture medium. Plates were cen-

trifuged (50g, 1 minute) and further incubated at 37°C. After 4 hours, 100 µl supernatant was collected from each well and counted in a gamma counter. NK-activity was expressed as percentage maximum lysis by detergent, both corrected for spontaneous ^{51}Cr-release. Recombinant IL-2 (500 U/ml) (Cetus, Emeryville, CA) was present during the entire assay to increase NK-activity.

Clinical pilot study

The first patients were treated in a single center, non-randomized, open-labeled, dose-escalating study (ongoing) with the aim of obtaining estimates of the safety and efficacy of the IT-combination administered to patients with life-threatening GVHD. Patients with acute GVHD were *eligible* if they had received second-line high-dose corticosteroid therapy (methylprednisolone 1000 mg/d) for at least 3 days without decrease in the severity of clinical symptoms. Patients were *not eligible* if they had evidence of intrapulmonary disease (which might aggravate the clinical severity of vascular leak syndrome [VLS][13]), or had allergy or antibodies to mouse Ig or dgA. Before entering the trial, all patients gave informed consent in accordance with the institutional review board and ethics committee. For assessment of safety and responses, patients were evaluated daily during hospitalization, and weekly thereafter, with a physical examination and by obtaining serum chemistries, complete blood counts and leukocyte differential. In addition, blood samples were collected for determination of the pharmacokinetics and immunogenicity of the IT-combination. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC; version 2.0).

IT-combination administration

Patients were supposed to receive 4 doses of the IT-combination administered intravenously in 4-hour infusions at 48-hour intervals. Prior to therapy, an intravenous test dose of 200 µg IT-combination was administered to rule out anaphylactoid reactions. Immunosuppressive agents used for prophylaxis and initial treatment of GVHD were allowed to remain unchanged.

Pharmacokinetics

Plasma levels of intact IT were measured with an enzyme immunoassay. Affinity-purified rabbit antiricin antibody (Sigma) in carbonate buffer (pH 9.6) was adsorbed to 96-wells maxisorp plates (Nunc, Roskilde, Denmark), and residual binding sites were blocked with 4% bovine serum albumin (BSA). Patient plasma samples were serially diluted in 50% PHS in the absorbed microtiter plates and incubated at 37°C for 1 hour. Subsequently, plates were washed and alkaline-phosphatase conjugated goat anti-mouse IgG2b or IgG2a (SBA, Birmingham, MI) was added at 37°C for 1 hour to bind captured SPV-T3a-dgA or WT1-dgA, respectively. Plates were washed, and the reaction was developed using p-nitrophenylphosphate. Optical densities were read at 450 nm, and levels of circulating IT were calculated from standard curves obtained with a known concentration of IT-combination diluted in pretreatment plasma. The detection limit

was 0.02 μg/ml for both SPV-T3a-dgA and WT1-dgA. Plasma concentration versus time data were analyzed using the nonlinear least square regression program WinNonlin version 1.1 (Scientific Consulting, Apex, NC).

Measurement of HAMA and HARA

An enzyme immunoassay was used for detection of antibody responses to components of the IT-combination. Patient plasma samples were serially diluted (undiluted to 1:2,048) in phosphate-buffered saline with 1% human serum albumin in 96-wells maxisorp plates (Nunc) containing either adsorbed SPV-T3a, WT1, or dgA. Bound human antibodies were probed using alkaline phosphatase-conjugated goat antihuman IgG/IgM (H+L) antibodies (Jackson, West Grove, PA), and the reaction was developed with p-nitrophenylphosphate. Optical densities were read at 450 nm, and the serum titer was expressed as the end-point dilution. Positive titers were considered to be at least 3 times background.

Flow Cytometry

Lymphocyte phenotyping was performed by multicolor flow cytometry using directly fluorescent-labeled MoAb according to a whole blood lysis method (FACS lysing, Becton Dickinson, San Jose, CA). T cells and NK cells were identified simultaneously with a mixture of fluorescence-labelled CD2 and CD5 MoAb (MT910-PE and DK23-PE, respectively) (DAKO, Copenhagen, Denmark). The percentage T/NK cells relative to all leukocytes was determined by costaining with a CD45-FITC MoAb (J33-FITC) (Immunotech, Marseille, France), and converted to absolute numbers based on the leukocyte count. B cells were quantified accordingly, using a PE-conjugated CD19 MoAb (HD37-PE) (DAKO).

Staging of GVHD and Definitions of Clinical Responses

Each organ system was staged grade 1 through 4 acute GVHD according to established criteria.[14] Patients were also given an overall grade of acute GVHD (I through IV) based on severity of organ involvement.[14] Responses to therapy were defined as follows. A complete response (CR) as the disappearance of symptoms in all organ systems. A partial response (PR) as the improvement of 1 organ or more, with no worsening in other organs. A mixed response (MR) as the improvement of 1 organ or more, with worsening in 1 other organ or more. Stable disease (SD) as no significant change in any organ system. Progressive disease (PD) as progression in 1 organ system or more without improvement in other organs.

RESULTS

In vitro elimination of T cells

T cells may become more susceptible to dgA-IT on activation.[15] This would be beneficial for the treatment of immunological disorders. Ideally, such treatment inhibits ongoing or beginning immune responses without affecting the resting T cell pool with its ability to counter future hazardous infections. To address this issue, nonactivated and PHA-activated PBMC were treated with IT for 24 hours and analyzed by flow cytometry for the number of surviving cells (Figure 1). Without prior PHA activation, treatment with SPV-T3a-dgA and WT1-dgA, either alone or in combination, resulted in a 2.0- to 2.7-fold reduction of viable cells at 10^{-8}M (about 1.8 μg/ml, the highest nontoxic concentration *in vitro*). After 48 hours PHA-activation, SPV-T3a-dgA, WT1-dgA, and the combination demonstrated an approximate 3-, 11-, and 34-fold increase in maximal killing capacity, respectively. The combination appeared very effective at concentrations above 10^{-9}M, resulting in the elimination of 99% of activated T cells at 10^{-8} M. Costaining with lineage-specific markers revealed no difference in vulnerability between $CD4^+$ and $CD8^+$ T-cell subsets (data not shown). Neither incubation with isotype-matched control IT, nor with MoAb SPV-T3a or WT1, significantly reduced the number of viable cells (< 1.15-fold reduction at 10^{-8} M; n = 3, data not shown).

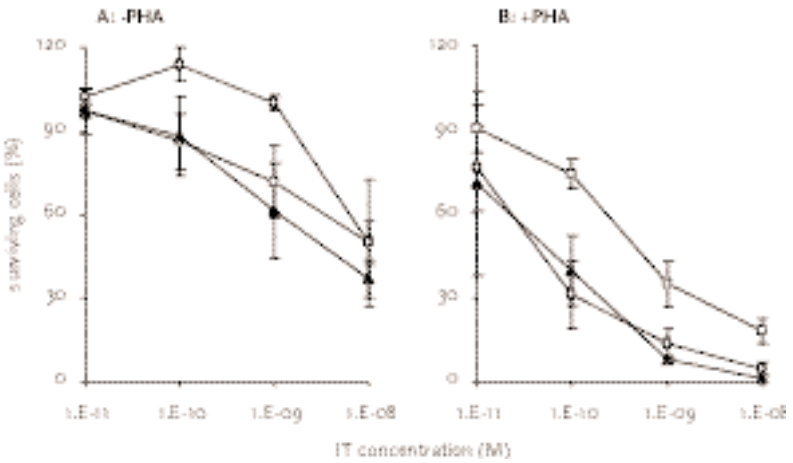

Figure 1 *Surviving T cells after treatment with IT*

Nonactivated (A) and PHA-activated (B) T cells were eliminated by SPV-T3a-dgA (❑) and WT1-dgA (❍), applied individually and in combination (▲)(half a dose each). Nonactivated and PHA-activated PBMC were incubated with various concentrations IT for 24 hours at 37°C. Following treatment, cells were washed and cultured for an additional four days in the presence of PHA to enable full exposure of IT toxicity. Subsequently, cells were stained with viability markers and analyzed by flow cytometry for the number of viable T cells relative to the untreated control. Data represent the mean ± SD as obtained with the PBMC of 3 healthy individuals.

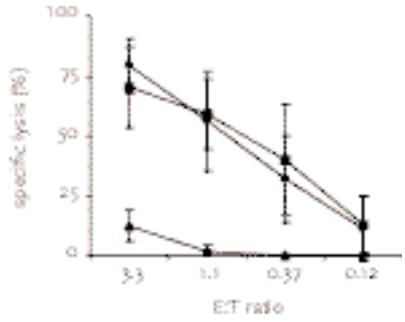

Figure 2
Reduction of CTL cytotoxicity by native MoAb SPV-T3a

An EBNA3C-reactive CTL clone was incubated with 10^{-8}M MoAb SPV-T3a (▲), isotype-matched irrelevant MoAb MOPC-141 (●) or culture medium (■) for 24 hours at 37°C. Subsequently, cells were washed and cultured for another 72 hours in culture medium. CTL cytotoxicity was then assayed by specific lysis of a ^{51}Cr-loaded autologous EBV-LCL, and expressed as percentage relative to maximum lysis by detergent. Data represent the mean ± SD of 3 experiments performed in triplicate.

Immunosuppressive activity of native MoAb SPV-T3a

SPV-T3a may deliver an immunosuppressive effect, irrespective of its conjugation to dgA, by modulation of the T-cell-receptor/CD3-complex (TCR/CD3-complex) or by induction of Fas-mediated apoptosis according to the process described as activation-induced cell death (AICD).[16] To mimic the alloactivation induced in a transplantation setting, a CTL clone reactive with minor histocompatibility antigen 'EBV-peptide EBNA3C' was stimulated with an autologous EBV-LCL. Incubation of this CTL with MoAb SPV-T3a for 24 hours, abrogated almost completely its cytolytic activity as measured in a ^{51}Cr-release assay (Figure 2). Flow cytometric analysis (Figure 3) revealed that this may be attributed to both (1) AICD of the CTL-clone (to 16% of the untreated control) and (2) modulation of the CD3 antigen (to 20% of the untreated control). In addition, about half of the non-modulated CD3 antigens was still occupied by SPV-T3a.

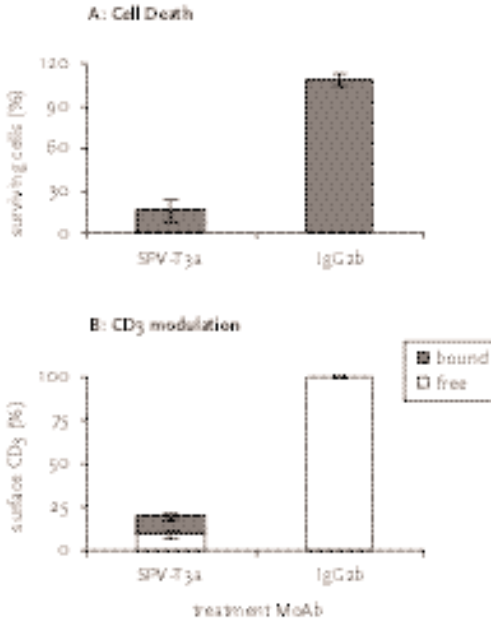

Figure 3
AICD and CD3-modulation by native SPV-T3a

The EBNA3C-reactive CTL clone was incubated with 10^{-8}M MoAb SPV-T3a, or isotype-matched control MoAb MOPC-141, for 24 hours at 37°C. Subsequently, cells were washed and cultured for another 72 hours in culture medium. The number of surviving cells was then determined by viability staining and flow cytometric analysis, and expressed as percentage relative to the untreated control (A). Membrane expression of free and SPV-T3a-bound CD3-antigen was determined as described in "Material and Methods" and indicated as percentage relative to control cells (B). Data represent the mean ± SD of 3 experiments performed in triplicate.

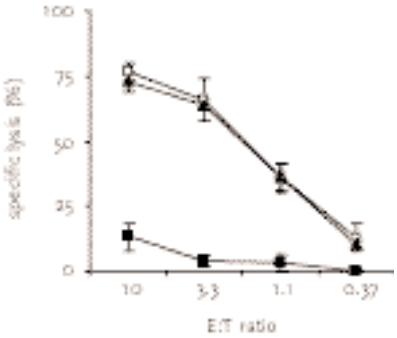

Figure 4
Effect of IT treatment on NK activity

PBMC were treated with 10^{-8}M WT1-dgA (■), SPV-T3a-dgA (▲) or without IT (❑), for 24 hours at 37°C. After treatment, cells were washed and cultured for an additional 3 days in culture medium without IT. Subsequently, remaining NK activity was determined by specific lysis of ^{51}Cr-labeled K562 blasts and expressed as percentage relative to untreated cells. During the experiment, 50 U/ml recombinant IL-2 was added to the culture medium to increase NK activity. Data represent the mean ± SD of 3 experiments performed in triplicate.

NK activity after IT treatment

Although initiated by CTL, GVHD is thought to be aggravated by less specific cytokine-stimulated "bystander cells" like NK cells.[3,17,18] The capacity of the described MoAb or IT to reduce NK activity was assayed by inhibition of lysis of ^{51}Cr-loaded K562 cells. Figure 4 shows the NK activity of PBMC incubated with IT for 24 hours. As expected, treatment with SPV-T3a-dgA did not effect the NK-activity (NK cells being $CD3^-$). In contrast, NK activity was almost completely abolished following 3 days after incubation with WT1-dgA. Similar results were obtained with cell line Daudi, which is predominantly vulnerable for lymphokine-activated killer cells (LAK cells; data not shown). Neither nonconjugated MoAb WT1, nor isotype-matched control IT UPC-10-dgA, impaired the lysis of K562 or Daudi cells (data not shown, n = 3).

Table 2 *Patient characteristics*

	Patient No.			
	1	2	3	4
Age /Sex	65/M	34/M	47/M	49/M
Primary disease[a]	MM	CML	MDS	MM
Primary treatment[b]	- M, P	- H, a-I	- none	- V, A, D
Transplant procedure[c]	- I, C, TBI, PSCT	- ATG, C, TBI, BMT	- I, C, TBI, BMT	- C, TBI, BMT,DLI
Staging of GVHD[d]	Liver: 3	Liver: 0	Liver: 0	Liver: 4
	Gut: 1	Gut: 0	Gut: 2	Gut: 0
	Skin: 4	Skin: 4	Skin: 0	Skin: 0
	Overall: IV	Overall: III	Overall: II	Overall: IV
Previous GVHD Medication[e]	C, Pl, S	C, Pl, S	C, Pl, S	C, Pl, S
IT-combination	2, 2, 4 mg/m^2	2, 2, 4, 4 mg/m^2	4, 4, 4, 4, mg/m^2	4, 4, 4 mg/m^2

a MM indicates multiple myeloma; CML: chronic myeloid leukemia; MDS: myelodysplastic syndrome.
b M indicates melphalan; P: prednisone; H: Hydrea, a-I: α-interferon; V: vincristine; A: Adriamycine; D: dexamethason.
c I: indicates idarubicine; C: cyclophosphamide; TBI: total body irradiation; PSCT: allogeneic peripheral stem cell transplantation; ATG: antithymocyte globuline; BMT: allogeneic bone marrow transplantation; DLI: donor lymphocyte infusion.
d Each organ system was staged grade 1 through 4 aGVHD according to Glucksberg et al.[14] Patients were also given an overall grade of aGVHD (I through IV) based on severity of organ involvement.[14]
e C indicates cyclosporine A; Pl: prednisolone; S: Solu-medrol (up to 1g daily for at least 3 days).

Pilot-study Participants

So far, 4 patients, all white men, have been enrolled in the study. The clinical features of these patients are summarized in Table 2. Patients 1 and 2 have been treated at the lowest dose level (2 x 2 mg/m^2 followed by 2 x 4 mg/m^2) and patient 3 and 4 at the second dose level (4 x 4 mg/m^2). Due to their early deaths, patient 1 and 4 received only 3 of the 4 planned infusions.

Table 3 *Pharmacokinetic parameters determined over the complete treatment course*

Patient No. (dose, mg/m2)	IT	$T_{1/2}$ (h)	AUC (μg·h/ml)	Cl (L/h)	V_d (L)	C_{max} (ng/ml)
1	SPV-T3a-dgA	3.4 ± 0.6	5.6 ± 0.6	0.36 ± 0.04	1.8 ± 0.2	1,830
(2, 2, 4)	WT1-dgA	5.5 ± 1.2	5.3 ± 0.8	0.37 ± 0.06	3.0 ± 0.3	1,380
	IT-Combination	4.0 ± 0.7	10. 5 ± 1.3	0.19 ± 0.02	1.1 ± 0.1	3,210
2	SPV-T3a-dgA	6.5 ± 1.0	3.4 ± 0.3	0.59 ± 0.06	5.6 ± 0.5	720
(2, 2, 4, 4)	WT1-dgA	6.7 ± 1.4	3.9 ± 0.5	0.5 ± 0.07	5.0 ± 0.6	930
	IT-Combination	6.6 ± 1.2	7.3 ± 0.8	0.28 ± 0.03	2.6 ± 0.3	1,650
3	SPV-T3a-dgA	6.9 ± 0.8	9.5 ± 0.7	0.64 ± 0.05	6.4 ± 0.4	675
(4, 4, 4, 4)	WT1-dgA	9.1 ± 1.4	3.4 ± 0.3	0.58 ± 0.06	7.6 ± 0.6	545
	IT-Combination	6.9 ± 0.8	12.4 ± 0.9	0.32 ± 0.02	3.2 ± 0.2	1,220
4	SPV-T3a-dgA	9.2 ± 1.5	12.2 ± 1.1	0.32 ± 0.03	4.4 ± 0.4	910
(4, 4, 4)	WT1-dgA	8.8 ± 2.7	7.5 ± 1.3	0.54 ± 0.09	6.8 ± 1.2	668
	IT-Combination	9.1 ± 1.9	19.7 ± 2.3	0.20 ± 0.02	2.7 ± 0.3	1,560

$T_{1/2}$, plasma half-life; AUC, area under the concentration versus time curve; Cl, clearance; V_d, volume of distribution; and C_{max}, maximum plasma concentration.

Pharmacokinetics

The plasma clearance curves best fitted a 1-compartment model with a constant rate infusion and a first-order elimination rate for both SPV-T3a-dgA and WT1-dgA individually and given in combination. The pharmacokinetic parameters determined over the entire courses are listed in Table 3. The mean $T_{1/2}$ was 6.5 ± 2.4 hours for SPV-T3a-dgA, 7.5 ± 1.7 hours for WT1-dgA, and 6.7 ± 2.1 hours for the IT-combination. Peak plasma levels were attained directly following each infusion and decreased (nearly) to baseline level in about 48 hours (Figure 5). The maximum plasma concentration (C_{max}) for the IT-combination ranged from 258 ng/ml following the first dose of patient 2 (2 mg/m^2) to 3,210 ng/ml following the third dose of patient 1 (4 mg/m^2). The latter seemed to be an exception as the maximum peak plasma level of the other patients fell within the relative narrow range of 1,220 to 1,650 ng/ml (all attained after a 4 mg/m^2 dose). The 2-fold higher level of patient 1 may be explained by his aggravating multi-organ failure interfering with the IT plasma clearance. When comparing the separate infusions, the mean $T_{1/2}$ of the IT-combination almost doubled over the complete treatment course (Table 4). This may be explained by a reduction of available target antigens, which act as an antigen sink especially during the first infusion(s).

Figure 5 *Plasma clearance curves*

IT-combination plasma clearance curves are shown from patient 1 (treated with 2, 2, and 4 mg/m^2) (○); patient 2 (2, 2, 4, and 4 mg/m^2) (□); patient 3 (4, 4, 4, and 4 mg/m2) (s); and patient 4 (4, 4, and 4 mg/m^2) (×). IT-combination plasma concentrations were deduced by summation of the individual values as determined for SPV-T3a-dgA and WT1-dgA.

Safety

The adverse events noted during the first 6 weeks following initiation of therapy are listed in Table 5. Because of the advanced disease of the patients, the etiology of the observed clinical events was obscured by multiple medical complications and concomitant medications. Patient 1 and 4 died during therapy due to worsening of complications already existing before start of the treatment, patient 1 from progressive multiorgan failure, patient 4 to generalized aspergillosis in combination with a cytomegalovirus (CMV) infection. Those symptoms thought to be related to the IT-combination were mild and transient. Patient 1 demonstrated edema in his shoulder not associated with weight gain, potentially due to limited capillary leakage. Patient 3 experienced episodes of fever during and between the IT-combination infusions. This fever may have been caused by a viral infection as well (see biological responses). Patient 4 demonstrated a rise of plasma CK-levels (not accompanied by myalgia) to 280 U/l on day 9 of therapy, being 1.45 times the upper limit of the normal range for men.

Table 4 *The $T_{1/2}$ (mean ± SD) determined over the separate infusions*

	Infusion No.			
	1 (n=4)	2 (n=4)	3 (n=4)	4 (n=2)
SPV-T3a-dgA	4.5 ± 0.8	5.4 ± 0.7	6.5 ± 2.8	6.2 ± 1.4
WT1-dgA	3.2 ± 1.5	8.3 ± 4.8	7.9 ± 2.0	9.0 ± 3.3
IT-Combination	3.8 ± 1.3	5.7 ± 1.5	7.1 ± 2.2	7.1 ± 0.4

Table 5 *Adverse Events observed during the first 6 weeks of therapy*[a]

	Total no. Grades 3/4 adverse events	All adverse events likely related to IT-combination
Allergy/Immunology	0	1[b]
Audiotory/Hearing	0	0
Blood/bone marrow	0	0
Cardiovascular	0	1[c]
Coagulation	1[d]	0
Constitutional symptoms	2[e]	1[b]
Dermatology/skin	0	0
Endocrine	0	0
Gastrointestinal	0	0
Hemorrhage	0	0
Hepatic	1[f]	0
Infection/febrile neutropenia	2[g]	0
Lymphatics	0	0
Metabolic/Laboratory	0	0
Musculoskeletal	0	1[h]
Neurology	1[i]	0
Ocular/visual	0	0
Pain	0	0
Pulmonary	0	0
Renal/Genitorurinary	0	0
Sexual/reproductive	0	0
Syndromes	0	0

a CTC-NCI criteria (version 2.0), using the bone marrow transplantation-specific scaling when applicable.
b Episodes of fever during and between infusions
c Limited capillary leakage
d TTP
e 40% decrease Karnofsky (n=2)
f Transient rise of SGOT & SGPT (after day 24)
g Aspergillosis, CMV-infection
h Rise in CK-levels to 1.45 times upper normal value
i Transient aphasia (<1 hour), probably related to TTP

The increase was not accompanied by a rise in the heart muscle isomer creatine kinase MB (CK-MB). Patient 4 had aphasia of short duration (<1 hour) 1 day after the first infusion of the IT-combination. At that time he was taking cyclosporine and had hemolysis with high reticulocyte counts, progressive thrombocytopenia, and fragmented red cells in the blood smear. It was concluded that aphasia was associated with cyclosporin-induced thrombotic thrombocytopenic purpura (TTP) and was not caused by the IT-combination (aphasia has been reported as a side-effect of other immunotoxins[19-21]). Cyclosporine was withdrawn and aphasia did not recur despite the further administration of the IT-combination.

Immunogenicity

Patient plasma samples were measured frequently before, during and after the trial for human-anti-mouse and human-anti-ricin antibodies (HAMA and HARA, respectively). No antibodies against SPV-T3a nor WT1 were detected in any of the cases. Patient 4

demonstrated a weakly positive HARA titer, 8 times the pretreatment value, on day 8 (one day before his death).

Biological Responses

Biological responses were monitored by flow cytometric evaluation of circulating T cells and/or NK cells. The concentrations of NK cells were too low to enable a reliable quantification with NK-specific markers. Therefore, NK cells and T cells were identified simultaneously using a mixture of a fluorescent-labeled CD2 (binding T cells and NK cells) and a CD5 MoAb (binding T cells). Figure 6 shows the amount of circulating T/NK cells expressed as percentage relative to the concentration at start of therapy. Patient 2 could not be adequately monitored due to the low initial number of circulating T/NK cells (about $6x10^7$/l). All 3 evaluable patients already demonstrated a remarkable reduction of T/NK cells during the first administration of IT-combination. Immediately after the 4 hours of infusion, the number of circulating T/NK cells of patient 1, 3, and 4 had dropped to 17%, 8% and 24% of the pretreatment level, respectively. For patient 1 and 4, this number gradually declined to 1 to 3 % after the third infusion. Patient 3, in contrast, showed signs of a rebound of NK/T cells between the infusions. After the last infusion, a progressive expansion of T/NK cells was observed resulting in

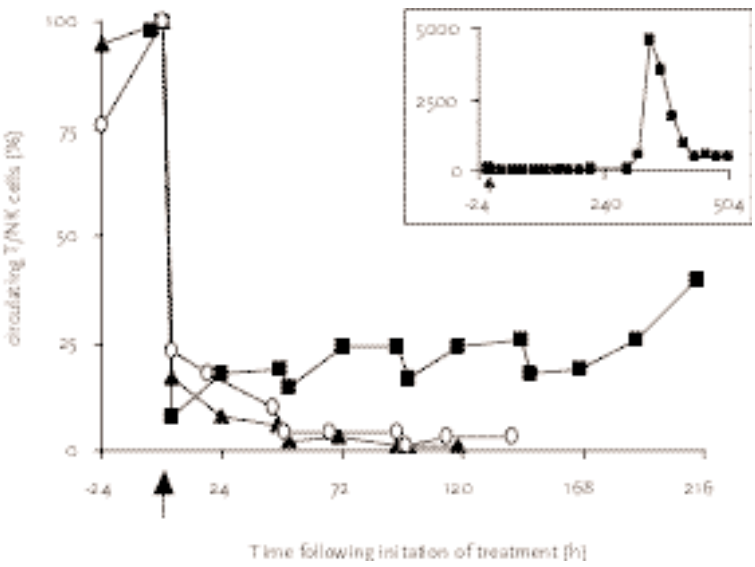

Figure 6 *Number of T/NK cells after IT-combination treatment*

Patient 1 (▲) and 4 (❍) received 3, and patient 3 (■) received all of the 4 planned IT-combination doses, given as 4-hour infusions at 48-hour intervals. The arrow beneath the x axis indicates the start of the first infusion. T/NK cells were identified by being $CD2^+$ and/or $CD5^+$. Their number was expressed as percentage relative to the concentration at start of therapy (being 0.7, 1.0, and 0.2 $x10^9$ cells/l for patient 1, 3, and 4, respectively). The inlay figure represents the amount of T/NK cells of patient 3 as determined over a longer period.

a peak concentration of about 45 times the pretreatment value at day 14. Flow cytometric analysis with CD4/CD8 MoAb and a T-cell receptor Vβ-panel pointed out that these cells were virtually all $CD8^+$ and oligoclonal of origin. At day 18, the number of T cells had decreased again to about 5 times the pretreatment level (probably by apoptosis as suggested by annexin staining, data not shown). These observations, combined with the episodes of fever patient 3 experienced during the first week, are suggestive for a T-cell response after a viral infection.

The slow B-cell repopulation following stem cell transplantation prevented a reliable quantification of circulating B cells in patients 1, 2, and 3. The concentration of peripheral blood B cells of patient 4 showed a normal biological fluctuation ($0.1\text{-}\ 0.2 \times 10^9$/l), which was not affected by the IT-combination. Moreover, the complete blood counts and leukocyte differential of all patients revealed no toxicity towards any of the other hematopoetic lineages (data not shown), thereby illustrating the selectivity of treatment for T/NK cells.

Skin biopsy specimens from patient 2 clearly demonstrated the biological efficacy of the IT-combination treatment (Figure 7). The microscopic appearance before treatment is typical for severe acute GVHD, with scattered infiltration of lymphocytes and a severely affected epidermal basal layer. After treatment, no remaining signs of lymphocyte infiltration could be detected, and the basal epidermis had regained its natural well-organized configuration. The intestinal GVHD of patient 3 was confirmed by a biopsy from the colon performed at day -5 (grade 3). When repeated at day 31, biopsy demonstrated a marked reduction of lymphocyte infiltrates without further signs of active GVHD. In patient 4 only a post mortem liver sample could be taken. Microscopic analysis revealed extensive cholestasis. Notably, no infiltrating lymphocytes could be detected.

Clinical Responses

Patient 1 showed a slight but clear improvement of his skin, from grade 4 to 3, starting at day 4. His (abnormal) liver functions remained stable; his gastrointestinal tract could not be objectively monitored due to administration of morphine. After 7 days of therapy, he died as a consequence of multiorgan failure. Patient 2 showed a dramatic reduction of his grade 4 GVHD of the skin to grade 0/1, starting 4 days after the first infusion and lasting for about 1.5 month. He then developed a modest relapse, GVHD grade 2 of the skin, which responded well to standard therapy of relatively low-dose corticosteroids supplemented with UV-B radiation. Patient 2 died 8 months after the IT-combination administration due to generalized aspergillosis and toxoplasmic encephalitis. Patient 3 showed strong improvement of his intestinal GVHD, grade 2 to grade 0, within 7 days after initiation of therapy. Apart from a mild and controllable GVHD reaction of his skin, grade 1 to 2 responding on low-dose corticosteroids, he showed no further signs of active GVHD. Two months after initiation of therapy, patient

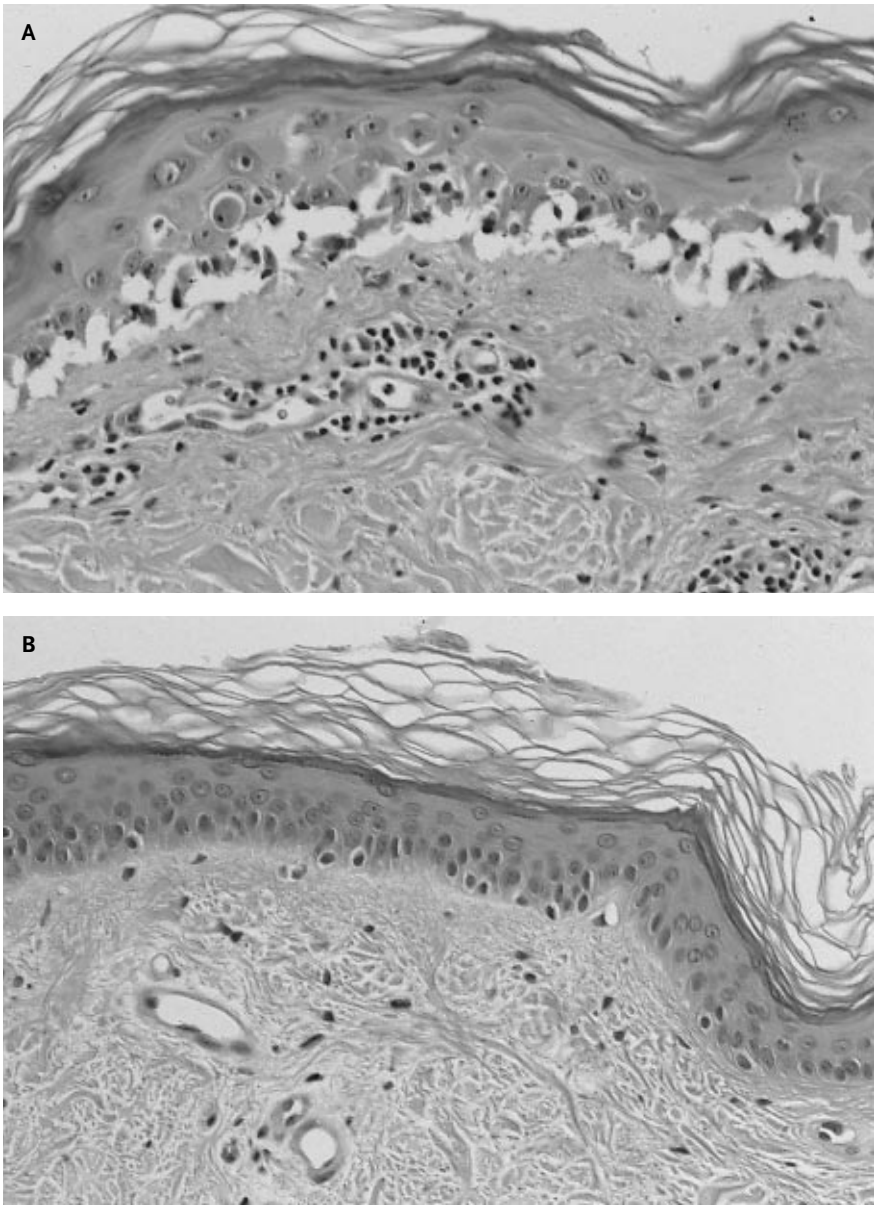

Figure 7 *Skin biopsies specimens*

Skin biopsies were obtained form patient 2 taken just before (A), and two weeks after (B) treatment with the IT-combination. The appearance of the skin before treatment is typical for severe GVHD. A scattered infiltration of lymphocytes can be observed, localized primarily around the blood vessels and the junctional region of dermis (lower side) and epidermis (upper side). The epidermal basal layer is destroyed and the epidermis is disrupted from the basal lamina. Following treatment, no remaining signs of lymphocyte infiltrations were detectable. The basal layer had regained its natural well-organized configuration (original magnification x100, hematoxylin-eosin)

3 died of a bacterial infection. Patient 4 showed a more or less stable GVHD of his liver during the 7 days of treatment. He died due to a generalized aspergillosis in combination with a CMV infection on day 8.

DISCUSSION

The *in vitro* efficacy data demonstrate that simultaneous application of SPV-T3a-dgA and WT1-dgA results in a synergistic cytotoxicity, leading to an approximately 99% elimination of activated T cells. A comparable synergism has been described for other IT combinations as well.[15,22-28] The most obvious advantage above single IT-treatment is that fewer target cells will be negative for multiple antigens than for a single antigen. In addition, the cells that express substantial levels of multiple target antigens may be loaded with more IT molecules. When the respective IT follow a different intracellular routing, the chance to escape the cytotoxic activity of the IT may be further reduced. With respect to anti-T-cell IT, reports addressing the combination approach are thus far focused on *in vitro* applications, including the purging of bone marrow grafts.[15,22-24] In this report, we state that the combination of SPV-T3a-dgA and WT1-dgA appears appropriate for the *in vivo* elimination of unwanted T cells as well. This particular combination affords important benefits that surpass the "common synergism" as observed with the combinations of anti-T cell IT described so far.

SPV-T3a was selected as CD3 MoAb based on its IgG2b-isotype which strongly reduces the risk of cytokine release syndrome.[29,30] The presence of a CD3-IT provides instant immunosuppression independent of dgA-based cytotoxicity (Figures 2 and 3). This may be of vital importance *in vivo* during treatment of acute life-threatening situations such as refractory GVHD. The limited (AICD) as well as temporal nature (blocking and modulation of CD3/TCR) of the underlying mechanisms provide arguments for making a "real killer" of SPV-T3a by conjugating it to dgA. The inclusion of the CD7-IT is essential, apart from the above-mentioned synergism, because it makes NK cells a target for the IT-combination as well (Figure 4). Accumulating evidence points at a distinctive role of NK cells in the pathophysiology of GVHD.[17,18]

The pioneer work of Vitetta and colleagues was of great value in designing the clinical pilot-study. They thoroughly studied the *in vivo* efficacy of RFB4-dgA (CD22) and HD37-dgA (CD19) in treating patients with non-Hodgkin Lymphoma in relation to different dose regimens.[13,20,21,31-33] In the present study, four patients with refractory acute GVHD have been treated with individual doses of 2 or 4 mg/m^2 IT-combination, according to their intermittent bolus infusion protocol. The mean $T_{1/2}$ of SPV-T3a-dgA and WT1-dgA resembled those of RFB4-dgA and RFB5-dgA (6.1-7.8 hours), the latter

being a CD25-IT for the treatment of Hodgkin lymphoma.[34] The blood clearance of these IT is more than twice as fast than reported for HD37-dgA ($T_{1/2}$ of 18.2 hours). As with RFB4-dgA and RFB5-dgA, the clearance appeared to be influenced by circulating target cells. In contrast to RFB4-dgA and RFB5-dgA, this did not result in large inter-patient variations. Instead, the $T_{1/2}$ of the IT-combination increased about 2-fold during the entire treatment course, probably due to (1) the rapid elimination of "MoAb-capturing" target cells (Figure 6), and/or (2) by a reduction of available binding sites per cell (modulation/occupation). The latter possibility especially holds true for CD3 (see also Figure 3b). The CD7-antigen, in contrast, demonstrates a rapid re-expression of free antigen, resulting in the restoration of pre-treatment expression levels within 48 hours (*in vitro* and *in vivo* observations, data not shown). Three patients suffered from severe GVHD that was more or less restricted to the skin, gastrointestinal tract, and liver, respectively. Their relative narrow-ranged $T_{1/2}$ and C_{max} demonstrates that the type of organ involved has no major impact on the pharmacokinetics. The single exception was the patient suffering from a generalized GVHD (affecting skin, liver and gastrointestinal tract) in combination with a severe kidney failure. His about 2-fold higher C_{max} may reflect a different IT handling due to his worsening multiorgan failure.

In three patients, C_{max} exceeded 1.2 to 1.6 times and in 1 patient C_{max} exceeded 3.5 times the "clinically save threshold level" as defined for RFB4-dgA and HD37-dgA (1 μg/ml).[13,20,32] Drug-related toxicities appeared to be restricted to limited capillary leakage and a modest rise of CK-levels (both n = 1). This may be the result of the IT-combination encompassing two distinct IT, each having a different spectrum of side effects (resulting in a distribution and, thereby, reduction in severity of drug-related toxicities). This is in agreement with observations of Vallera *et al*.[35] and Onda *et al*.[36], who both reported on comparable IT (directed against the same target cell/antigen and containing the same toxin), that displayed different toxicity profiles when tested in the same mouse model. None of the 4 patients treated with the IT-combination developed detectable HAMA. Only 1 patient showed a positive HARA titer (8 times baseline level). Apart from the small population size, this may be attributed to the huge dosages of corticosteroids administered, and to incomplete reconstitution of the B cell compartment early after transplantation.

The design of the pilot study does not allow an estimation of long-term toxicities (*e.g.* increase in opportunistic infections or secondary malignancies). Three of the 4 patients treated so far eventually died of an infection. The IT-combination can not be excluded as a contributing factor to the underlying immunosuppression. However, a realistic long-term safety profile can only be obtained when the IT-combination is applied without extensive preceding and concomitant second-line medication. At least theoretically, based on its short $T_{1/2}$ and narrow specificity range, the IT-combination is expected to accomplish a relatively limited immunosuppression by only temporarily affecting the T/NK cell compartment. Moreover, the composition of the IT-combination is better

defined and expected to vary less than that of ATG/ALG[37-39], which use is often associated with sustained T cell depletion.[40,41] From this viewpoint, it appears encouraging that the third patient showed a strong oligoclonal T-cell expansion directly after treatment with the IT-combination. The presumed antiviral T-cell response was not associated with a relapse of GVHD. This suggests a strong immunogenicity of the respective antigen and/or some form of treatment selectivity towards activated cells. The latter is in agreement with the *in vitro* observations and might be explained by the fact that on activation T cells show an increased expression of the CD7 target-antigen[9,10] and become vulnerable for CD3-triggered AICD.[42,43]

All patients showed a response, ranging from the elimination of the majority of circulating T/NK cells, to the dramatic clinical responses as observed in the patients 2 and 3. The biphasic pattern of T/NK cell removal indicates that multiple mechanisms are involved, some of which have not been observed *in vitro*. As for the *in vitro* efficacy data, the IT-combination needs several days before it displays its full killing capacity. This is in accordance with observations of Beyers *et al*.[5], who reported that it took 5 daily infusions of H65-ricin A (CD5) to achieve a more or less gradual reduction of T cells to 20% of the initial number. In contrast, the IT-combination induced *in vivo* a massive and rapid reduction of T/NK cells already during the first four hours of infusion. Comparable biological responses have been described for native murine MoAb[44-46], including anti-CD3/TCR MoAb of IgG2b isotype.[47,48] The underlying mechanism is not fully clear yet. Some indirect evidence is provided that MoAb SPV-T3a is responsible for this initial rapid T-cell reduction. Tax and colleagues (unpublished observation, W.J.M.T.) demonstrated that nonconjugated MoAb WT1 did not influence the number of circulating T cells when administered *in vivo* to renal patients for the treatment of graft rejection. In addition, flow cytometric analysis of blood samples of the first patient demonstrated that the minor population of $CD3^-CD7^+$ cells (mostly NK cells) was reduced at a much slower rate (up to 2 days) than the $CD3^+CD7^+$ T cells (data not shown). In general, reduction of lymphocytes by native murine MoAb is often transient, a few days or less. In the present study, the initial rapid elimination was followed by a more gradual but sustained reduction of T/NK cells. This suggests a dgA-based elimination of target cells, which is supported by the *in vitro* killing capacity of the IT-combination observed at concentrations obtained *in vivo*.

It is impossible from the clinical data to determine the exact contribution of SPV-T3a-dgA and WT1-dgA (or their MoAb-moieties) to the observed biological and clinical responses. Unfortunately, no animal models are available that allow an efficacy evaluation of the individual components (SPV-T3a does not bind monkey-CD3). The high tolerability of the IT-combination argues against the clinical testing of theoretically less effective individual components (being single IT or MoAb). Instead, further studies should be focussed on elucidating the full potency of the IT-combination in its present form. Apart from dose optimization and application in an earlier phase of GVHD, this

may include the treatment of alternative indications that potentially benefit from the *in vivo* elimination of T cells.

The authors are aware of the limited nature of the clinical data obtained so far. However, the major finding that emerged from these data is that the IT-combination concept might work. Substantial biological as well as clinical responses have been observed in the absence of acute severe toxicities. This is especially meaningful considering the extensive pretreatment of the enrolled patients. Ideally, further studies will point out that the IT-combination forms a safe and effective tool for helping control certain diseases mediated by T cels.

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CHAPTER 8

SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

This thesis addresses the use of ITs in relation to hematological disorders. **Chapter 1** provides a general overview of the mechanisms of action and current status of ITs, whereas **Chapter 2** focuses on the potential applications in the field of hematology. The subsequent **Chapters 3 to 5** describe part of the institutional fundamental research performed to improve this form of therapy. In **Chapters 6 and 7**, the rationale as well as the 'in house' preparation and first clinical results of an IT-combination for GVHD are addressed. Hereinafter, **Chapters 3 to 7** are discussed in more detail followed by a general perspective regarding the current and future status of ITs.

Chapter 3 describes a comparison of the internalization and cytotoxicity of an anti-CD3 ricin A chain IT (WT32-RTA). The study is based on an improved method to determine the cellular uptake of radiolabeled MoAb/IT. Initially, internalization studies were solely relying on cell-associated radioactivity. The activity removable by acid or protease treatment formed a measure for membrane bound MoAb/IT, whereas non-removable activity represented the amount of internalized molecules. Though very useful in studying several IT-related phenomena, this method had a major shortcoming. Intracellularly processed (read: degraded) and subsequently exocytosed MoAb/IT was not taken into account. With regard to IT, however, these molecules could very well have contributed to the intoxication of the cell by delivering the toxin before being processed. When studying internalization, it is therefore important to measure the amount of processed and shed MoAb/IT-fragments in the culture supernatant as well. This was hampered, initially, by the presence of other low weight radioactive material in the starting material. Removal of this material was cumbersome, involving dialysis or repetitive dilution/concentration steps of the radioactive starting material. Some investigators circumvented this problem by preloading target cells with radiolabeled MoAb/IT at 4°C, and subsequently washing out small radioactive particles and unbound MoAb/IT. The actual internalization studies could then be performed at 37°C, with intracellularly

processed and shed MoAb/IT now being readily detectable as the low weight radioactive material emerging in the supernatant. A limitation of this strategy, however, was the absence of unbound MoAb/IT surrounding the target cells. Normally, this excess of MoAb/IT influences the MoAb-antigen binding equilibrium, and forms a 'sink' for the binding of re-expressed antigen. With the introduction of dispensable concentrators, the extensive removal of low weight radioactive material came within practical reach (to <0.3% of the total radioactivity). Consequently, internalization studies could be performed in the continuous presence of unbound MoAb/IT and more accurately reflected the therapeutic situation.

When thus applied to study the internalization of the CD3 MoAb/IT with leukemia T-cell line Jurkat, an ongoing biphasic cellular uptake of MoAb/IT could be demonstrated. This internalization was characterized by a rapid initial uptake, followed by slower but progressive internalization driven by the constant re-expression of CD3-antigen. Addition of cytotoxicity enhancers NH_4Cl or monensin retarded intercellular degradation, resulting in an increased number of intracellularly present IT/MoAb. This should increase the possibility of cytoplasmatic deliverance of the toxin-moiety and, thereby, lower the amount of internalized IT necessary for intoxication of the cell. These observations closely matched the kinetics of cytotoxicity as determined in a protein synthesis inhibition assay. Both prolongation of the incubation time and addition of NH_4Cl or monensin clearly increased the efficacy of the CD3-IT towards Jurkat cells. An interesting observation was that the IT-induced cytotoxicity was characterized by a lag period due to a delay in either the internalization or the subsequent processing of membrane bound IT. Translated to the *ex vivo* purging of bone marrow, the above results argue for a 24 h IT-incubation in the presence of either NH_4Cl or monensin to ensure an optimal purging efficacy, also to cells with low antigen density, without causing harm to normal progenitor cells.

Looking at a future role of internalization studies, they may be of help in determining optimal dose schedules for *in vivo* treatment. They could provide a rationale for selecting either a continuous or bolus infusion protocol, depending on their effect on internalization. When considering multiple bolus infusions, internalization studies may help to identify the optimal interval. Increasing the time between infusions, for instance, may render target cells more vulnerable for the subsequent dose by allowing a more complete re-expression of antigen.

Another example of the relevance of internalization studies is described in **Chapter 4**. This study addresses the striking discrepancy in cytotoxicity of two anti-CD19 ricin A-ITs differing in isotype only (IgG1 and IgG2a). Internalization experiments with both anti-CD19 MoAb, as well as with their $F(ab')_2$ fragments, revealed that the presence of the IgG1 Fc-part enhanced the cellular uptake. The involvement of FcγRII (CD32) in this process was demonstrated by a decreased cytotoxicity of the IgG1-IT (and not IgG2a-

IT) in the presence of FcγRII-blocking MoAb. These results suggest that the IgG1 Fc-part of membrane bound anti-CD19 IT interacts with FcγRII, which supports the cellular uptake of the antigen-IT complex analogous to the mechanism described by Kurlander. Additional experiments identified functional FcγRIIa as the responsible FcγR-isoform. The tremendous increase in potency as compared to the relative modest rise in internalization (~100-fold and 3-fold, respectively), suggests that the enhanced uptake is accompanied by a different intracellular routing which favors ricin A toxicity. Alternatively, the increased number of intracellular molecules may exceed a critical threshold above which the cell is no longer capable of efficiently inactivating the IT. The relevance of this phenomenon for other ITs has to be determined yet. Additional experiments with an IgG1 anti-CD22 IT revealed no effect of FcγRII-blocking on cytotoxicity. It should be noted, though, that the intracellular routing of anti-CD22 ITs is more effective than that of anti-CD19 ITs (as indicated by a superior toxicity despite similar or lower internalization rates). Any favorable effect of the IgG1-isotype might therefore only marginally influence the already high efficacy of anti-CD22 ITs. Nevertheless, the above study forms a clear example of the potential importance of the MoAb isotype on IT efficacy. This will be even more apparent regarding *in vivo* applications, considering the potential interactions with the patient's immune system. As such, these data provide an argument against the routine exclusion of MoAb Fc-fragments from recombinant fusion toxins. Though being technically more convenient, Fv-based ITs lack the (potential) benefit of Fc-mediated effector mechanisms contributing to, or even synergizing, the toxin-based elimination of target cells.

Prior to clinical use, ITs have to be extensively screened for activity against target and non-target cells. **Chapter 5** describes a sensitive flow cytometric (FCM) method capable of determining IT-induced cell kill in freshly derived patient-samples. This method is based on the FCM determination of surviving target-cells following staining with viability markers propidium iodine (PI) and calceine (Calc). The exact quantification of surviving cells was enabled by admixture of a known concentration of inert beads prior to FCM-analysis. An important finding using this method was that IT-based inhibition of protein synthesis is not directly accompanied by the physical disruption of the cell membrane. A post-treatment period of several days was necessary to enable full exposure of the IT-induced cell kill, comparable to that as determined with clonogenic assays.

As demonstrated in Chapter 5, the described method enables the evaluation of target cell depletion in a heterogeneous population as blood or BM. The use of a cell-specific, fluorescence-labeled, MoAb is most straightforward, enabling live/death discrimination and target-cell identification in one analysis. For this approach to be useful, the target-cell needs to express a specific antigen besides the target-antigen already used for therapy. The latter is inappropriate as this antigen might be modulated or covered by the therapeutic MoAb/IT. Moreover, residual malignant cells may be enriched for cells

with low levels of target-antigen expression. When no appropriate 'identification-antigen' is present, target cells may be identified by PCR-amplification of a cell specific DNA-sequence. This requires the pre-sorting of the viable fraction to prevent the DNA of IT-eliminated cells from contributing to the PCR-signal. In practice, triple-sorted viable cells should contain less than one in 10^6 erroneously sorted non-viable cells, matching the detection limit of a sensitive PCR-assay.

The flowcytometric analysis of cell kill has now been applied in various studies evaluating the efficacy of varying IT towards different cell types, including freshly derived heterogeneous samples.[1,2] These studies confirmed the relevance of directly measuring the number of surviving cells, and suggest this assay may prove a helpful tool in optimizing IT-therapy. As such, it forms a valuable addition to the conventional clonogenic and protein synthesis inhibition assays.

The **first part of Chapter 7** describes the experiments that provided the rationale for the development of an IT-based therapy for aGVHD. These experiments pointed out that anti-CD3 and anti-CD7 ricin A-IT (SPV-T3a-dgA and WT1-dgA, respectively), act synergistically in killing T cells. The most obvious advantage of using a combination of ITs is that fewer target cells will be negative for multiple antigens than for a single antigen. Moreover, cells expressing both target-antigens may be loaded with more IT molecules. Apart from this 'common synergism', the particular combination of SPV-T3a-dgA and WT1-dgA affords important additional benefits. The presence of a CD3-IT provides instant immunosuppression, which relies on the binding of the CD3/T-cell-receptor complex (CD3/TCR) and is independent of dgA-based toxicity. The underlying mechanisms, modulation of the CD3/TCR complex and activation induced cell death (AICD), may be of vital importance *in vivo* during treatment of acute life-threatening situations such as refractory GVHD. The anti-CD3 MoAb SPV-T3a was selected based on its 'non-T-cell-activating' IgG2b-isotype which strongly reduces the risk of cytokine release syndrome (associated with the use of 'activating' CD3 MoAb). Inclusion of the CD7-IT is essential, apart from the above mentioned 'common synergism', since it makes NK cells (largely $CD3^-/CD7^+$) a target for the IT-combination as well. Accumulating evidence points to a distinctive role of NK cells in the pathophysiology of aGVHD. Altogether, these results formed the base for the clinical evaluation of the IT-combination in patients with refractory aGVHD.

The conversion of laboratory experiments to the early clinical studies asks for a revolutionary change of the production process. Apart from the scale up of equipment and materials, the handling, purity and activity of the final product have to meet stricter requirements. It would be most straightforward to outsource such productions to authorized contract manufacturers with relevant experience. Unfortunately, the required financing often forms a major obstacle. Even early clinical studies are generally considered 'too less fundamental' to qualify entirely for regular scientific granting. Potential

pharmaceutical partners, on the other hand, often prefer clear signs of clinical efficacy before taking the obvious risk of investing in experimental drugs. To overcome this impasse, and supported by a grant of the government (technology foundation STW), it was decided to perform most of the production and validation 'in house'. **Chapter 6** describes this production of clinical grade material as performed at the Central Hematology Laboratory under supervision of the department of Clinical Pharmacy, and with the assistance, amongst others, of the departments of Pathology, Virology and the Central Animal Laboratory. To ensure the highest possible safety and functionality of the final product, previously a draft quality was formulated based on the FDA-document 'Points to consider in the manufacture and testing of monoclonal antibody products for human use'. Reports of the production of comparable ITs already tested in FDA-approved trials formed an additional guideline. The actual production provided sufficient material for the planned clinical pilot study. Yields were comparable to those previously reported in literature (>25% MoAb-recovery). The specifications of the final products mostly matched those as prescribed in the draft-quality. The few minor deviations, regarding the percentage unconjugated WT1 and the DNA-content of both WT1-dgA and SPV-T3a-dgA, were, retrospectively, considered to be within an acceptable range. Animal experiments revealed a murine LD_{50} (lethal dose for half of the animals) equal, or higher, than that of similar ITs already in clinical use. The major concern formed the cross-reactivity of SPV-T3a with the human esophagus, observed during the *in vitro* screening of a human tissue panel. To evaluate the potential clinical relevance of this observation, human doses IT-combination were administered to two cynomolgus monkeys (sharing the *in vitro* cross-reactivity). Neither toxicity nor binding of SPV-T3a-dgA to the monkey esophagus could be detected. Notably, therapeutic serum concentrations (>1.8 μg/ml) were obtained for several hours in both monkeys without acute irreversible toxicities occurring. The main side effect was a transient rise of CK-levels, indicative for dgA-associated muscle injury. Altogether, the above data justified the evaluation of the produced material in a clinical pilot-study for the treatment of life-threatening aGVHD. As such, the 'in house' production can be considered an essential stepping stone towards the future large-scale productions required for the actual registration studies.

So far five patients have been treated with three to four infusions of 2 or 4 mg/m^2 IT-combination, administered intravenously at 48-hour intervals. The **second part of Chapter 7** describes the results obtained with the first four patients. The $T_{1/2}$ was ~7 hours, with peak serum levels ranging from 258 to 3,210 ng/ml. The infusions were well tolerated. Drug-associated side effects were restricted to limited edema, fever, and a modest rise of creatine kinase levels, suggesting the maximal tolerated individual dose lies well above 4 mg/m^2. Remarkably, the IT-combination did not provoke vascular leakage, or other serious toxicities, while the peak serum concentrations in all patients either reached (n = 1) or exceeded (n = 4) the 'clinically save threshold' as defined for

comparable IT (being 1 μg/ml). An explanation for this may be that administration of two distinct ITs may result in a distribution and, thereby, dilution of individual side effects. This is in accordance with observations of others who reported on almost identical ITs (directed against the same target cell/antigen and containing the same toxin), which displayed different toxicity profiles when tested in the same mouse model. Moreover, the concomitant administration of high dose corticosteroids may have positively affected the maximal tolerable dose. Notably, only one of the patients treated so far developed low titer antibodies against ricin A. This may again be attributed to the high dose corticosteroids. Alternative explanations are the incomplete reconstitution of the B cell compartment early post-transplantation, and the successful depletion of accessory T cells. Biological responses could be noted in all five patients, as characterized by a rapid and extensive drop of circulating T/NK cells. The biphasic pattern of this depletion suggests the involvement of multiple mechanisms. The rapid depletion of T cells, already observed directly after infusion, appears to be MoAb-related. While the subsequent more gradual but sustained elimination of T/NK cells points to a ricin A-based mechanism. Noteworthy, one patient mounted an extensive CD8 T-cell response directly following treatment (indicative for an anti-viral immune response), not accompanied with aggravating GVHD. The other patient whose peripheral T cells could be reliably monitored for a longer period, demonstrated a gradual reconstitution of circulating T cells starting two weeks after initiation of therapy. The accompanying shift in the TCR-Vβ repertoire, suggested these were newly generated T cells. (patient No. 5, unpublished observations). Two of the five patients already died during the first treatment week, due to underlying diseases and pre-existing organ impairment. The three other patients demonstrated extensive clinical responses, despite unresponsiveness to the intensive pretreatment.

Though limited in nature, these preliminary data are suggestive for a clear therapeutic window where, in contrast, hardly one existed for the other dgA-based IT tested so far. This may be explained by the combination approach, which, on one hand, decreases the overall toxicity, and, on the other hand, increases effectiveness by appealing on multiple, synergistic, effector mechanisms. Notwithstanding the clear biological and clinical responses, all five patients eventually died due to transplantation related complications (mostly infections). These results are inherent to the type of study, only permitting the enrollment of patients with very advanced and therapy-resistant disease (not responding to cyclosporin plus 1g corticosteroids daily). The absence of severe side effects, now justifies the treatment of a limited number of patients with less advanced aGVHD. This enables the evaluation of the IT-combination in a more appropriate clinical setting. Moreover, it should predict the suitability of the IT-cocktail for alternative indications that may benefit from the temporary removal of T/NK cells. Most obvious is the treatment of graft-rejection (either stem cells or solid organs). In addition, animal studies clearly demonstrated that anti-T cell IT may have a role in creating the prop-

er immunologic environment for non-myeloablative stem cell transplantation, achieving tolerance induction, or even treating certain T-cell mediated auto-immune disorders.

General perspective

The introduction of a new therapeutic strategy often creates high expectations regarding its use for treating life-threatening diseases. These do not always reflect the inevitable hurdles generally accompanying the development cycle of new drugs. As such, ITs form no exception. Based on the initial reports, describing complete cures of lethally tumor-xenografted mice, they seemed the ultimate representation of Paul Ehrlich's magic bullet concept. The subsequent translation of ITs to the clinic, however, revealed major obstacles preventing instant therapeutic success in man. Several of the problems encountered resembled those previously seen in MoAb trials. Obvious examples form the occurrence of humoral responses, in this case also directed against the toxin-moiety, and the heterogeneous expression of target-antigens. Moreover, as with MoAbs, the Phase I/II testing in patients with advanced bulky disease is not considered optimal to expose the full potential of this form of treatment. Most importantly, though, the clinical studies revealed serious toxicities, not previously detected to the same extent in the test animals. Especially for ricin-based ITs, the occurrence of severe vascular leakage dramatically narrowed the therapeutic window. As a consequence, safely attainable serum concentrations appeared to be several-fold lower than those previously determined to be effective in mice.[3] Altogether, these early clinical results tempered the initial hype, leaving only a limited number of old-time believers still trying to make the concept work. Basically, their approach was to transfer ITs back to appropriate animal models, and to solve the individual obstacles with the help of new exciting (recombinant-DNA) techniques coming available.[4-6] As it looks now, there are some serious indications that their persistence will be rewarded.

With respect to reduction of the immunogenicity, various strategies are currently being explored. One is based on the conjugation of non-immunogenic human toxin equivalents to either human antibodies, growth factors or cytokines. This approach already resulted in the construction of several 'fully human' ITs with proven *in vitro* activity[7-12]. Another strategy focuses on making the highly potent conventional toxins less immunogenic by genetically altering the most immunogenic determinants, or by pegylation of the molecule.[13,14] Combined with routinely produced human MoAbs, these may render 'Stealth' ITs being (almost) invisible for the patient's immune system. A third approach is based on transient repressing immune reactions by the co-administration of immunomodulating agents.[15,16] In order to anticipate on heterogeneous antigen expression, the simultaneous use of multiple ITs have been addressed. Several animal studies convincingly demonstrated the synergistic efficacy of a cocktail of ITs.[17-19] Additionally, the first clinical results obtained with such combinations are now being

reported.[2,20] Noteworthy, the IT-combination described in Chapters four and five underscored the potential significance of MoAb-based effector mechanisms contributing to the overall IT efficacy.[2] The superiority of the concomitant use of ITs with conventional chemo/radiation therapy has been clearly demonstrated in several animal experiments.[21-23] These encouraging results should facilitate the clinical evaluation of ITs in the more appropriate adjuvant setting. With regard to the dose limiting toxicities, the most important finding is probably the unraveling of the cause of vascular leakage. Baluna *et al*. of the group of Vitetta identified a sequence motif in the ricin A chain, present in other toxins and the VLS-inducing cytokine IL-2 as well, which is responsible for VLS by binding to and damaging endothelial cells.[6,24-27] Future studies should point whether deletions or mutations in this sequence, or the use of nondamaging blocking peptides, may increase the therapeutic index of ITs prepared with the conventional plant or bacterial toxins.

Altogether, these recent developments will undoubtedly result in the production of next generation ITs with strongly improved *in vivo* characteristics. It appears that ITs are still very much alive and ready to fulfill at least some of their clinical promises in the foreseeable future. As such, the recent FDA-approval of a recombinant IT for the treatment of a refractory hematological malignancy (Ontak, DAB389IL-2), might be regarded as an important and indicative turning point.

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SAMENVATTING

Immunotoxines (ITs) zijn hybride moleculen bestaande uit een zogenaamde monoclonale antistof (MoAb) gekoppeld aan een effectieve gifstof (toxine). De gebruikte MoAbs hebben de eigenschap dat ze zeer sterk, en met een hoge mate van specificeit, binden aan een eiwit (antigeen) dat voorkomt op de buitenkant van de te vernietigen cel. Eenmaal in de bloedbaan ingespoten, zullen de ITs zich aan deze cellen kunnen binden. Het IT-antigeen complex wordt vervolgens door de cel opgenomen (geïnternaliseerd), waarna het toxine zijn werk kan doen en de cel zal uitschakelen (zie Figuur 1, Hoofdstuk 1). Daar de gebruikte toxines op eigen kracht de cel niet binnenkomen, treffen ITs in principe alleen die cellen waartegen het MoAb gericht is.

Het UMC St Radboud introduceerde 15 jaar geleden als een van de eerste research centra het IT onderzoek in Nederland. De eerste klinische toepassing betrof de zuivering van autologe beenmergtransplantaten van patiënten met een kwaadaardige bloedziekte. Doel van deze behandeling was de eventueel aanwezige kankercellen in het beenmergtransplantaat op te ruimen om daarmee de kans op terugkeer van de kwaadaardige ziekte te verkleinen. De min of meer routinematige toepassing van deze behandeling kan beschouwd worden als het startpunt van dit proefschrift. Deze toepassing leidde op zichzelf tot additioneel onderzoek om de mate van opzuivering van het beenmerg te verbeteren, alsook om deze op een betrouwbare mannier te monitoren. Internationaal leidde het enthousiasme over de mogelijke therapeutische toepassingen ondertussen tot diverse (meest Amerikaanse) studies waarbij ITs aan patiënten werden toegediend voor de bestrijding van diffuse en solide tumoren en uiteenlopende immuunziekten. Aangemoedigd door de behaalde resultaten, speciaal op het gebied van beenmerg- en lymfeklieraandoeningen, en ingegeven door de klinische behoefte, besloot de afdeling Hematologie een op IT gebaseerde therapie te ontwikkelen voor omgekeerde afstoting na beenmergtransplantatie (graft-versus-host disease: GVHD). Dit proefschrift beschrijft een deel van het IT onderzoek zoals dat gedurende de laatste tien jaar in het UMC St Radboud is uitgevoerd. Na een algemene inleiding (**Hoofdstuk 1**) en de positionering binnen de hematologie (**Hoofdstuk 2**) volgt een beschrijving van het fundamentele laboratorium onderzoek (**Hoofdstukken 3 en 5**) en de opzet en eerste klinische resultaten van een pilot-studie voor de behandeling van GVHD (**Hoofdstukken 6 en 7**). In het hierop volgende worden Hoofstukken 3 tot en met 7 in meer detail besproken, gevolgd door een algemene beschouwing over de huidige en toekomstige rol van ITs.

Hoofdstuk 3 beschrijft een verbeterde methode om de cellulaire opname te bepalen van radioactief gelabelde MoAbs of daarvan afgeleide ITs. Het bijzondere aan deze methode is dat ze bij de bepaling van de totale internalisatie rekening houdt met moleculen die intracellulair zijn afgebroken en vervolgens door de cel zijn uitgescheiden. Dit

is met name van belang voor ITs, waarbij dergelijke moleculen kunnen bijdragen aan de intoxicatie van een cel door het toxine af te leveren alvorens te worden afgebroken.

Bovenstaande methode werd gebruikt om de relatie tussen internalisatie en effectiviteit van een anti-CD3 IT (WT32-RTA) te bestuderen. Met behulp van leukemische T-cellijn Jurkat, kon een voortdurende cellulaire opname van het anti-CD3 MoAb/IT worden gedemonstreerd. Deze werd gekenmerkt door een snelle initiële opname gedurende de eerste 12 uur, gevolgd door een langzamere maar voortdurende internalisatie gedreven door constante reëxpressie van het CD3-antigeen. Toevoeging van NH_4Cl of monensine vertraagde de intracellulaire afbraak, wat resulteerde in een toegenomen hoeveelheid intracellulair aanwezig IT/MoAb. Theoretisch leidt dit tot een verhoogde kans op afgifte van het toxine, en daarmee een verlaging van het aantal geïnternaliseerde moleculen benodigd voor de intoxicatie van de cel. Deze observaties kwamen sterk overeen met de daadwerkelijke effectiviteit van het anti-CD3 IT zoals bepaald in een eiwitsynthese inhibitie assay. Zowel een verlenging van de incubatietijd, alsook de toevoeging van NH_4Cl of monensine, leidde tot een verhoogde cytotoxiciteit ten aanzien van Jurkat cellen. Een interessante observatie hierbij was dat de maximale eiwitsynthese remming iets leek na te lopen, ten gevolge van een vertraging in de internalisatie ofwel daaropvolgende verwerking van membraangebonden IT. Vertaald naar de zuivering van beenmerg, pleiten bovenstaande resultaten voor een 24-uurs incubatie met IT in de aanwezigheid van NH_4Cl of monensine. Dit zou resulteren in een optimale effectiviteit, ook ten aanzien van cellen met lage antigene dichtheid, zonder schade te berokkenen aan normale hematopoietische progenitorcellen.

Voor wat betreft de toekomst, kunnen internalisatiestudies van pas komen bij de bepaling van het optimale doseringsschema voor klinische studies. Zij zouden een onderbouwing kunnen leveren voor protocollen met een continue dan wel meerdere kortdurende infusies, afhankelijk van het effect op de internalisatie. In het geval van kortdurende infusies kunnen zij bovendien van nut zijn voor de bepaling van het optimale interval tussen twee opeenvolgende infusies. Verlenging van dit interval, bijvoorbeeld, kan leiden tot een meer volledige reëxpressie van het doelwit-antigeen, en daarmee, een verhoging van de gevoeligheid voor een volgende dosis.

De relevantie van internalisatie studies wordt onderstreept in **Hoofdstuk 4**. Deze studie behandelt het opmerkelijke verschil in effectiviteit tussen twee anti-CD19 ITs die alleen verschillen in MoAb-isotype (IgG1 en IgG2a). Internalisatie studies met zowel intact IgG als $F(ab')_2$ fragmenten, brachten aan het licht dat de aanwezigheid van het anti-CD19 IgG1-Fc gedeelte resulteert in een verhoogde cellulaire opname. De rol van Fcγ-receptor II (FcγRII: CD32) in deze kon worden aangetoond door een verlaagde effec-

tiviteit van het anti-CD19 IgG1-IT in aanwezigheid van FcγRII-blokkerende MoAb. Deze resultaten wijzen op een interactie van membraangebonden anti-CD19 IgG1-MoAb met FcγRII, resulterende in een verhoogde internalisatie van het antigeen-IT complex, naar analogie van het zogenaamde Kurlander-effect. Met behulp van additionele experimenten kon (functioneel) FcγRIIa vervolgens geïdentificeerd worden als het verantwoordelijke receptor-isotype. De relatief bescheiden toename in internalisatie gaat gepaard met een onevenredige verhoging van de cytotoxiciteit (ongeveer 3-voudig en 100-voudig, respectievelijk). Dit wijst erop dat de toename in internalisatie gepaard gaat met een afwijkende intracellulaire route gunstig voor de effectiviteit van het toxine, ofwel dat de hoeveelheid intracellulair IT een kritische grens overschrijdt waarboven ze niet meer effectief geïnactiveerd kan worden. De relevantie van dit fenomeen voor andere ITs is nog niet duidelijk. Additionele experimenten met een anti-CD22 IgG1-IT lieten geen positief effect zien van de blokkade van FcγRII op de cytotoxiciteit. Echter, de intracellulaire routing van anti-CD22 IT is veel effectiever dan die van anti-CD19 IT (gegeven de superieure toxiciteit bij gelijke of lagere internalisatie). Enige positieve effecten van het IgG1-isotype hebben daarom waarschijnlijk slechts een marginaal effect op de reeds hoge effectiviteit van het CD22-IT.

Bovenstaande *in vitro* studie geeft een duidelijk voorbeeld van het potentiële belang van het MoAb Fc-gedeelte op de uiteindelijke IT effectiviteit. Bij de daadwerkelijke klinische toepassing zal dit nog duidelijker naar voren komen, denkend aan de mogelijke interacties met het humane immuun systeem. Als zodanig vormen bovenstaande data een argument tegen het routinematig uitsluiten van Fc-fragmenten bij zogenaamde 'recombinante fusietoxines'. Deze missen de eventuele bijdrage van Fc-gebaseerde effector mechanismen aan de toxine-gebaseerde opruiming van ongewenste cellen.

Voor de ontwikkeling van een optimale IT therapie is het van belang het aantal ongewenste cellen te bepalen dat ontsnapt aan verschillende behandelingschema's. In **Hoofdstuk 5** wordt een gevoelige flowcytometrische methode beschreven om de IT-geïnduceerde celdood in verse patïentenmonsters op een adequate wijze te kunnen monitoren. Deze methode is gebaseerd op de flowcytometrische identificatie van dode en levende cellen na kleuring met viabiliteitsmerkers. De exacte kwantificering van overlevende cellen wordt mogelijk gemaakt door bijmenging van een bekende hoeveelheid inerte bolletjes voorafgaande aan de analyse. Een belangrijke bevinding bij het gebruik van deze methode was de totale omvang van de IT-geïnduceerde celdood pas na enkele dagen aangetoond kan worden.

Zoals gedemonstreerd in Hoofdstuk 5, kan met de beschreven methode de opruiming van ongewenste cellen in een heterogene populatie als bloed of beenmerg worden

bestudeerd. Het meest eenvoudige is het om voor de identificatie van deze cellen gebruik te maken van een fluorescerend celspecifiek MoAb. Dit vereist echter de expressie van een 'identificatie antigeen' anders dan het therapeutisch gebruikte 'doelwit antigeen'. Deze laatste kan gemoduleerd zijn of bezet met het therapeutische MoAb/IT. Daarnaast kunnen de overblijvende cellen verrijkt zijn voor cellen met een lage expressie van het doelwit antigeen. Als alternatief kan gekozen worden voor de PCR-amplificatie van een celspecifieke DNA-sequentie. Pre-sorting van levende cellen moet in dit geval voorkomen dat het DNA van dode maar nog aanwezige cellen ten onrechte bijdraagt aan het PCR signaal. In de praktijk leidt een 'triple-sort' tot minder dan één op de miljoen foutief gesorteerde dode cellen, wat overeenkomt met de detectielimiet van een gevoelige PCR assay.

De flowcytometrische bepaling van IT-geïnduceerde celdood is inmiddels toegepast in uiteenlopende studies, ondermeer voor de monitoring van IT-effectiviteit in heterogene, vers geïsoleerde, bloed- of beenmergmonsters van patiënten. Deze studies suggereren dat deze methode zeer bruikbaar is voor de optimalisering van IT-therapie, en een waardevolle aanvulling vormt op de conventionele assays.

Het **eerste deel van Hoofdstuk 7** beschrijft de experimenten die de onderbouwing leverden voor de ontwikkeling van een IT-gebaseerde therapie voor aGVHD. Deze experimenten wezen uit dat een anti-CD3 en anti-CD7 IT (SPV-T3a-dgA en WT1-dgA, respectievelijk), een synergistische werking vertonen ten aanzien van de eliminatie van T cellen. Een algemeen voordeel van het gebruik van een combinatie van ITs is dat minder cellen negatief zullen zijn voor twee dan voor één antigeen. Bovendien kunnen cellen die beide antigenen tot expressie brengen beladen worden met meer IT moleculen. Afgezien van dit 'gewone' synergisme, kent de combinatie SPV-T3a-dgA en WT1-dgA nog een aantal extra voordelen. Het CD3-IT levert additionele immunosuppressie door de binding van het CD3/T-cell-receptor (CD3/TCR) complex. De onderliggende oorzaken, modulatie van het CD3/TCR complex en activatie geïnduceerde celdood (AICD), kunnen van vitaal belang zijn tijdens de behandeling van acute levensbedreigende situaties zoals therapieresistente aGVHD. Het anti-CD3 MoAb (SPV-T3a) is geselecteerd op basis van zijn 'niet-activerende' IgG2b-isotype, welke het risico op 'cytokine release syndrome' sterk vermindert. De toevoeging van het CD7-IT is essentieel, afgezien van het bovengenoemde 'gewone' synergisme, omdat het ook NK cellen (grotendeels $CD3^-$ $CD7^+$) elimineert. Er zijn steeds meer aanwijzingen voor de rol van NK cellen in de pathofysiologie van aGVHD. Bovenstaande bevindingen vormden de wetenschappelijke basis voor de klinische evaluatie van de IT-combinatie in patiënten met therapieresistente GVHD.

De vertaling van laboratoriumexperimenten naar (vroege) klinische studies vraagt een grondige aanpassing van het productieproces. Afgezien van de opschaling van materialen en grondstoffen, moeten de bewerking, zuiverheid en activiteit van het eindproduct aan veel strengere eisen voldoen. **Hoofdstuk 6** beschrijft de productie zoals uitgevoerd op het Centraal Hematologisch Laboratorium onder supervisie van de afdeling Klinische Farmacie, en met de ondersteuning van, onder andere, de afdelingen Pathologie, Virologie en het Centraal Dierenlaboratorium. Om een zo hoog mogelijke veiligheid en functionaliteit van het eindproduct te garanderen, werd van te voren een ontwerpkwaliteit geformuleerd gebaseerd op het FDA-document 'Aandachtspunten in de vervaardiging en het testen van monoclonale antilichaam producten voor humaan gebruik'. Beschrijvingen van de productie en validatie van vergelijkbare IT die reeds getest waren in FDA-goedgekeurde trials vormden een additionele richtlijn. De 'in huis' productie leverde voldoende materiaal op voor de voorgenomen pilot-studie. De specificaties van het eindproduct kwamen grotendeels overeen met de ontwerpkwaliteit. De enkele uitzonderingen, aangaande het percentage ongebonden WT1 en de DNA-hoeveelheden van WT1-dgA en SPV-T3a-dgA, vielen, retrospectief, binnen aanvaardbare grenzen. De LD_{50} (lethale dosis voor 50% van de dieren) voor muizen, kwam overeen, of was zelfs hoger, dan die van vergelijkbare IT die reeds in de kliniek werden toegepast. De voornaamste zorg betrof de kruisreactiviteit van SPV-T3a met humaan oesophagus epitheel, waargenomen tijdens de *in vitro* screening van een humaan weefselpanel. Ter bestudering van de klinische relevantie van deze bevinding, werden humane doses IT-combinatie toegediend aan een tweetal cynomolgus apen. In deze studies kon geen toxiciteit noch binding van het SPV-T3a-dgA aan de oesophagus worden aangetoond. Therapeutische serumconcentraties (>1.8 μg/ml) werden in beide apen bereikt gedurende meerdere uren, zonder dat er onomkeerbare acute toxiciteiten optraden. De belangrijkste bijwerking was een tijdelijke verhoging van kreatine kinase (CK)-waarden, wijzend op spierafbraak door het toxine.

Bovenstaande data rechtvaardigden de evaluatie van het geproduceerde materiaal in een klinische pilot-studie voor de bestrijding van levensbedreigende aGVHD. De onderliggende 'in huis' productie kan wat dat betreft gezien worden als een essentiële stap richting de toekomstige grootschalige productie voor de daadwerkelijke registratiestudies.

Tot nog toe zijn vijf patiënten behandeld met drie of vier infusies van 2 of 4 mg/m^2 IT-combinatie, toegediend middels een vier uur durend intraveneus infuus met tussenpozen van 48 uur. De resultaten zoals behaald met de eerst vier patiënten staan beschreven in het **tweede deel van Hoofdstuk 7**. De gemiddelde serum halfwaardetijd was ongeveer 7 uur, met piek serumwaarden variërend tussen 258 tot 3.210 ng/ml.

Ondanks dat piek serumconcentraties werden behaald die gelijk (n = 1) of boven (n = 4) de klinisch veilige grens lagen zoals gedefinieerd voor vergelijkbare IT (1 μg/ml), werden geen ernstige toxiciteiten waargenomen. De bijeffecten waren beperkt tot een geringe vochtretentie (n = 1), koorts (n = 2) en een lichte stijging van CK waarden (n = 1), wijzend op een maximaal tolereerbare doses van 4 mg/m^2 of meer. Dit opmerkelijk gunstige toxiciteitsprofiel is waarschijnlijk terug te voeren op de toediening van twee verschillende ITs, welke mogelijk resulteert in een distributie en daarmee verdunning van individuele bijeffecten. Daarnaast kan de gelijktijdige toediening van hoge doses corticosteroïden de maximaal tolereerbare dosis positief beïnvloed hebben. Slechts één van de tot nog toe behandelde patiënten ontwikkelde een geringe antilichaamrespons tegen ricine A. Afgezien van de hoge dosering corticosteroïden, kan dit verklaard worden door een onvolledige reconstitutie van het B-cel compartiment en/of de succesvolle eliminatie van accessoire T-cellen door de IT-combinatie.

Alle vijf patiënten vertoonden een duidelijke biologische respons, gekenmerkt door een gefaseerde daling van circulerende T/NK cellen. De initiële snelle depletie van T-cellen, reeds gedurende de infusies, lijkt MoAb-gerelateerd, terwijl de daaropvolgende meer geleidelijke daling van T/NK cellen wijst op een ricine A gebaseerd mechanisme. Een patiënt ontwikkelde direct na de laatste infusie een sterke oligoclonale expansie van CD8-positieve T-cellen, niet gepaard gaande met een terugkeer van de GVHD (indicatief voor een anti-virale immuun reactie). De andere patiënt wiens perifere T-cellen voor een langere tijd gevolgd konden worden, liet twee weken na start van de behandeling een geleidelijke terugkeer van circulerende (waarschijnlijk naïeve) T-cellen zien. Twee van de vijf patiënten overleden voor toediening van het laatste infusie ten gevolge van reeds bestaande complicaties. De drie overige patiënten vertoonden een duidelijke klinische respons, ondanks dat hun ziekte niet gereageerd had op de voorafgaande behandeling.

Bovenstaande gegevens wijzen op een duidelijke 'therapeutische window'. Dit kan verklaard worden door de combinatie-benadering die gelijktijdig de toxiciteit vermindert en de effectiviteit verhoogt door een beroep te doen op meerdere, synergistische, effector mechanismen. Niettegenstaande de duidelijke biologische en klinische responsen, zijn alle vijf patiënten uiteindelijk overleden aan transplantatie gerelateerde complicaties (meest infecties). Dit is inherent aan het type studie, welke alleen de behandeling van patiënten met vergevorderde, therapieresistente, ziekte toestaat. De bemoedigende eerste klinische resultaten rechtvaardigen nu de evaluatie bij patiënten met minder vergevorderde GVHD. Daarnaast kunnen andere indicaties overwogen worden die baat zouden hebben bij de tijdelijke opruiming van T- en NK-cellen. Meest voor de hand liggend is de behandeling van afstoting na transplantatie (van stamcellen of solide orga-

nen). Afgezien hiervan hebben dierstudies overtuigend aangetoond dat anti-T-cel ITs de juiste immunologische omgeving kunnen creëren voor niet-myeloablatieve stamceltransplantatie, het realiseren van tolerantie-inductie, of zelfs de behandeling van bepalde T-cel gemedieerde autoimmuun-ziekten.

Algemene beschouwing

De introductie van nieuwe therapeutische strategieën roept dikwijls hooggespannen verwachtingen op ten aanzien van de behandeling van levensbedreigende ziekten. Deze worden vaak gevolgd door de onvermijdelijke tegenslagen die veelal gepaard gaan met de ontwikkeling van nieuwe medicijnen. ITs vormen hierop geen uitzondering. Volgend op de eerste publicaties, die de genezing beschreven van muizen getransplanteerd met levensbedreigende humane tumoren, werden ITs beschouwd als immunologische 'magic bullets'. Gedurende de daaropvolgende vertaalslag naar de kliniek, stuitte men echter op belangrijke obstakels die onmiddellijke therapeutisch successen in de weg stonden. Een aantal van deze obstakels was reeds waargenomen bij de vroege MoAb-studies. Duidelijke voorbeelden vormen het optreden van antilichaam-reacties, in dit geval tevens gericht tegen het toxine, en de heterogene expressie van doelwit-antigenen. Afgezien hiervan, lijkt de opzet van Fase I/II studies, met de inclusie van patiënten met therapie-resistente uitgebreide ziekte, niet optimaal voor de evaluatie van de potentie van MoAbs of ITs. De belangrijkste tegenvaller was echter het optreden van ernstige bijweringen welke voorheen niet in die mate bij proefdieren waren waargenomen. In het geval van ricine-gebaseerde ITs betrof dit met name ernstige 'vaatlekkage syndroom'. De hieruit volgende, relatief lage, maximaal tolereerbare doses resulteerden in maximale serumconcentraties die beduidend lager lagen dan de therapeutische concentraties waargenomen in de oorspronkelijke muizenmodellen. Alles bij elkaar temperden deze resultaten de oorspronkelijke hype, wat als gevolg had dat slechts een relatief klein groep van onderzoekers van het eerste uur overbleef om het concept nieuw leven in te blazen. De ITs werden hiertoe teruggebracht in het diermodel om daar de verschillende obstakels één voor één aan te pakken met de beschikbaar gekomen nieuwe revolutionaire technieken (o.a. recombinant DNA-technieken). Zoals het zich nu laat aanzien, lijkt dit doorzettingsvermogen beloond te worden.

Wat betreft de immunogeniciteit, worden momenteel verschillende strategieën toegepast. Een van deze is gebaseerd op de identificatie van humane toxine-equivalenten (b.v. humane RNases). Koppeling van dergelijke stoffen aan antilichamen, groeifactoren of cytokines van humane origine, resulteert in principe in hybride moleculen met lage of afwezige immunogeniciteit. Deze benadering heeft reeds geresulteert in de constructie van verscheidene 'volledig humane' ITs met bewezen *in vitro* activiteit. Een

andere strategie is gericht op de verlaging van de immunogeniciteit van de uiterst potente conventionele toxines. Dit kan ofwel door de genetische modificatie van de meest immunogene determinanten, ofwel door deze chemisch af te schermen met polyethyleen glycol ('PEGylation'). In combinatie met humane MoAb, zou het gebruik van dergelijke gemodificeerde toxines resulteren in zogenaamde 'Stealth ITs' welke nagenoeg onzichtbaar zijn voor het immuunsysteem van de patiënt. Als alternatief voor bovenstaande strategieën, bestaat er ook nog de mogelijkheid van een tijdelijke onderdrukking van immuunreacties door een gedoseerd gebruik van immuun-onderdrukkende medicijnen.

De aanpak van heterogene antigene expressie wordt gezocht in de gelijktijdige toediening van meerdere ITs gericht tegen verschillende antigenen. Het synergistische effect van dergelijke combinaties is inmiddels in verscheidene dierstudies op overtuigende wijze aangetoond. Op dit moment verschijnen publicaties van de eerste klinische resultaten verkregen met dergelijke combinaties. De IT-combinatie beschreven in Hoofdstukken 5 en 6 onderschrijft bovendien het belang van MoAb-gebaseerde effector mechanismen op de algehele effectiviteit van IT.

Verscheidene dierexperimenten hebben inmiddels overduidelijk het nut aangetoond van het gebruik van ITs als aanvulling op de conventionele chemotherapie of bestraling. Deze bemoedigende resultaten brengen de klinische evaluatie van ITs in de meer geëigende adjuvant setting dichterbij.

Wat betreft de dosis limiterende bijwerkingen, is de meest in het oog springende ontwikkeling waarschijnlijk de opheldering van de oorzaak van vaatlekkage syndrome (VLS). In de A-keten van ricine is een aminozuurketen volgorde (motief) geïdentificeeerd die ook aanwezig is in de andere conventionele plantaardige en bacteriële toxines alsook in het eveneens vaatlekkage inducerende Interleukine-2 (IL-2). Dit motief blijkt verantwoordelijk voor VLS door de binding en destructie van endothiale cellen. Naar verwachting zal de verwijdering of mutatie van dit motief, ofwel het gebruik van niet-destructieve blokkerende peptides, de therapeutische index van conventionele ITs sterk verhogen.

Het leidt geen twijfel dat de bovenstaande ontwikkelingen zullen resulteren in de productie van nieuwe ITs met sterk verbeterde *in vivo* karakteristieken. Alles wijst er dan ook op dat ITs levensvatbaarder zijn dan ooit, en binnen afzienbare tijd tenminste een deel van hun oorspronkelijke beloften in zullen lossen. De FDA-goedkeuring van een recombinant IT (Ontak, DAB389IL-2) voor de behandeling van een therapieresistente hematologische maligniteit, kan dan ook beschouwd worden als een belangrijke mijlpaal.

LIST OF ABBREVIATIONS

AICD	activation-induced cell death
ATG	antihymocyte globuline
AUC	area under the concentration versus time curve
BMT	bone marrow transplantation
BSA	bovine serum albumin
Calc	calcein AM
CD	cluster of differentiation
CK	creatine kinase
C_{max}	maximum plasma concentration
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
dgA	deglycosylated ricin A-chain
DLI	donor lymphocyte infusion
DLT	dose limiting toxicity
DT	diphtheria toxin
DTT	dithiothreitol
ELISA	enzym-linked immunosorbent assay
ER	endoplasmatic reticulum
Fab	antigen binding fragment of monoclonal antibody
Fv	variable fragment of Fab
FCM	flow cytometry
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FS	forward scatter
aGVHD	acute graft-versus-host disease
HAMA	human-anti-mouse antibodies
HARA	human-anti-ricin antibodies
HLA	human leucocyte-associated antigen
HRP	horseradish peroxidase
ID_{50}	50% inhibiting dose
IFN	interferon
IL	interleukin

Ig	Immunoglobulin
IT	immunotoxin
kDa	kilo Dalton
LD_{50}	50% lethal dose
MHC	major histocompatibility complex
MoAb	monoclonal antibody
MTD	maximal tolerable dose
NHL	non-Hodgkin's lymphoma
NK cell	natural killer cell
NO	nitric oxide
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PE	*Pseudomonas* exotoxin
pH	hydrogen ion strength
PHA	phytohaemagglutinin
PI	propidium iodide
RAS	right angle scatter
RIP	ribosome inhibitory protein
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMPT	4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene
SPDP	*N*-succinimidyl-3-(2-pyridyldithio)propionate
SD	standard deviation
STC	stem cell transplantation
TCR	T cell receptor
TNF	tumor necrosis factor
$T_{1/2}$	plasma half-life
TTP	thrombotic thrombocytopenic purpura
VH	heavy chain variable domain
VL	light chain variable domain
VLS	vascular leak syndrome
WBC	white blood cell count

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 30 mei 1964 te Nijmegen. In 1984 behaalde hij het Atheneum-B diploma aan het Elshof College te Nijmegen, waarna hij begon met de studie Biologie aan de Katholieke Universiteit Nijmegen. In het kader van deze studie werden stages gelopen bij de afdeling Parasitologie van het bedrijf Intervet te Boxmeer (Dr. A. Vermeulen), de afdeling Moleculaire Biologie van de Katholieke Universiteit Nijmegen (Prof. Dr. R.N.H. Konings†) en de afdeling Hematologie (Prof. Dr. C. Haanen, later Prof. Dr. T. de Witte) van het UMC St Radboud te Nijmegen. In september 1989 behaalde hij het doctoraal examen Biologie met als specialisatie 'Toegepaste Richting'. Vanaf die tijd is hij werkzaam op het Centraal Hematologisch Laboratorium (Dr. J. Wessels, later Dr. E. Mensink) van het UMC St Radboud, als wetenschappelijk onderzoeker op het door Dr. F. Preijers geïnitieerde Immunotoxine onderzoek. Gedurende dit onderzoek is het plan opgevat om de veelbelovende laboratorium resultaten te vertalen in een experimenteel medicijn, de zogenaamde immunotoxine-combinatie (IT-combinatie), voor patiënten met levensbedreigende omgekeerde afstotingsziekte (graft-versus-host disease: GVHD). Middels een subsidie van de Stichting Technische Wetenschappen (Technologiestichting STW) van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), en met een actieve ondersteuning door diverse afdelingen binnen het UMC St Radboud, heeft de schrijver een pilot-studie opgezet om de IT-combinatie op beperkte schaal in de kliniek te testen. De 'in huis' productie en daaropvolgende klinische toepassing vormen als zodanig de bekroning van het in dit proefschrift beschreven onderzoek. Op basis van de eerste hoopgevende resultaten is besloten de IT-combinatie verder bedrijfsmatig te ontwikkelen, waartoe in 1998 het aan het UMC St Radboud gelieerde 'Immunotoko B.V.' is opgericht. Dit initiatief is ondersteund door de NWO die de schrijver een subsidie heeft toegekend in het kader van het Stimuleringsprogramma voor Innovatief Geneesmiddelenonderzoek en Ondernemerschap in Nederland (STIGON). Met deze support gaat hij, als bestuurder van Immunotoko B.V., de komende periode trachten de IT-combinatie verder te ontwikkelen richting registratie als een 'Orphan Drug' tegen GVHD en afstoting van transplantaten.

De schrijver is getrouwd met Joyce Burgers en de trotse vader van Jelle, Youp en Merel.